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Cytotoxicity of a Horse Antihuman Cancer Serum for Normal and Malignant Cells *In Vitro* [1]

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SUMMARY

By our tissue-culture techniques we have demonstrated species-specific antibodies in Björklund's horse antihuman cancer serum absorbed with human plasma. No cancer-specific lytic antibody that could not be absorbed by normal tissues was detected. Absorption studies with cancer or liver tissues removed cytolytic activity for both normal and malignant cells. If absorption was incomplete, a drop in cytotoxic activity was reflected by a delay in cell damage. When absorption was ineffective, as with skeletal muscle, brain, or plasma, lysis occurred to both normal and cancer cells. In our experiments the Sillen antiserum did not reveal a cancer-specific antibody directed against an antigen characteristic of neoplasia.—J. Nat. Cancer Inst. 27: 487–501, 1961.

DURING THE period 1911–40, there were several reports of specific damage to cancer cells *in vitro* by anticancer serums (*1, 2*). The specificity of these reactions could not be confirmed (*3–7*). Improved techniques for propagation of cells of normal and malignant origin in continuous culture and better methods for testing cytotoxic effects *in vitro* have stimulated interest in more sensitive assays of immune serums prepared against neoplastic tissues.

A number of studies has described effects of anti-HeLa serum on HeLa and other cells of human origin growing *in vitro* (*8–12*). In general, our observations corresponded with those reported by others: Anti-HeLa cell serum prepared in rabbits lysed HeLa cells and other long-established human tissue-culture strains of malignant or normal origin (*12*). Furthermore, new cultures of normal leukocytes and leukemic leukocytes were lysed by any antihuman cell serum. Complement was required for rapid cell damage and eventual lysis.

We did additional experiments with antiserums prepared in rabbits against fresh normal and malignant human tissues obtained during surgery. Both antiserums caused lysis of human cells growing *in vitro*.

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[2] We thank David Pressman and Theodore S. Hauschka for their suggestions during preparation of the manuscript.

Absorption of these antiserums with sediments of normal human liver removed all the cytotoxicity toward the human cells grown *in vitro*. From these studies we concluded that antiserums prepared in rabbits against fresh normal or malignant human tissues or against human cells growing *in vitro* were merely species-specific (*12–15*) and lacked any demonstrable antibodies directed selectively against neoplastic cell constituents.

The recent experiments of Björklund and associates (*16–18*) were aimed at producing a specific antiserum in horses against human cancer cells. Björklund reported that such antiserums absorbed with human plasma lysed human cancer cells *in vitro*, but did not damage normal human cells grown under similar conditions. These investigators completely removed the cytotoxic effect of their serums by absorption with cancer cells, but there was no loss of antineoplastic activity when absorptions were made with normal tissues (*17*). In an attempt to confirm these observations with one of the anticancer (Sillen) serums prepared and used in Björklund's own laboratory, we used tissue-culture techniques that are highly sensitive for demonstrating cytotoxic antibodies.

MATERIALS AND METHODS

Horse antihuman cancer serum.—The sterile, lyophilized serum from an immunized horse, Sillen, bled on January 8, 1958, was kindly supplied by Dr. Bertil Björklund.[3] The contents of a vaccine vial, which contained 2.14 g dry weight of serum, were reconstituted to the original volume of 25 cc with sterile double-glass distilled water.

Absorption of antiserums.—The details of our method of absorption of antiserums with tissue homogenates have been described (*13*). In some experiments, sediments of normal human liver or normal rat liver were washed with medium 199 until a clear supernatant was obtained on centrifugation. Three cc of serum was then absorbed for 1 hour with 6 cc of wet, packed human liver or rat liver samples.

Portions (1.2 cc) of the Sillen antihuman cancer serum were absorbed with 100 mg of lyophilized, pooled human cancer, liver, skeletal muscle, or brain, as described by Björklund (*17*).

When the Sillen serum was added to the cancer tissue obtained from Björklund, it formed a dense granular homogenate and less than half the serum was recovered after centrifugation at 20,000 $\times$ *g* for 30 minutes at 2° C on the following day. The liver preparation absorbed less serum and was not as granular as the cancer tissue. The skeletal muscle and brain tissue formed fine homogenates and most of the serum was recovered from these preparations after centrifugation.

Umbilical cord obtained at term delivery was washed free of blood and cut into small fragments with scissors. The fragments were homogenized in a blendor at 4° in 15 cc of balanced salt solution for 4 minutes. The

[3] The Sillen antiserum and lyophilized human tissues for this work were obtained from Dr. David Pressman, of the Department of Biochemistry Research, Roswell Park Memorial Institute.

thick homogenate was lyophilized for 24 hours. Fifty mg of lyophilized umbilical cord was used to absorb 1.2 cc of the Sillen serum, as outlined by Björklund (*17*).

A portion of Sillen serum was absorbed overnight at 5° with an equal amount of fresh, pooled, citrated human plasma. During the first 2 hours the tube was frequently shaken. A precipitate, part of which was composed of fibrin clot, formed overnight. This serum mixture was centrifuged at 1000 rpm for 10 minutes, and the clear supernatant was diluted with Eagle's medium and tested against HeLa and normal fetal fibroblasts. The Sillen serum was also absorbed overnight in the cold with freshly pooled, lyophilized human plasma—the procedure described by Björklund. (Apparently, plasma is the only absorbent that makes the anticancer serum specific.)

Fresh, pooled guinea-pig serums were used to supply complement for the test systems. The guinea-pig serum produced no cytotoxic changes on any of the cells studied at the concentrations used.

All cell types tested against normal or Sillen horse serum were grown on glass in mediums containing either calf or human serum. Two hundred units of penicillin and 40 γ streptomycin per ml were routinely added to the culture mediums. The mediums were then replaced with the test serum in medium 199, with or without complement. No contamination occurred in the cultures during the observation period.

Cells Against Which Normal and Anticancer Horse Serums Were Tested

1) *Normal human amnion.*—Small fragments of human amnion were explanted on glass in Carrel flasks in a fluid medium (20% calf serums/80% Eagle's medium). Outgrowths of amnion cells and of fibroblasts developed after 4 or 5 days, and cells were tested on the 7th and 10th days after explantation.

2) *Normal human adult muscle.*—Fragments of muscle were explanted in cultures of chicken-plasma clot. The fragments developed dense outgrowths of spindle-shaped cells after 6 to 7 days. These cultures were trypsinized once and cells explanted on the glass surface of Carrel flasks, T-30 flasks, and on slides in a fluid medium composed of 20 percent calf serum and Eagle's medium. The cells were tested 10 and 12 days after explantation.

3) *Normal foreskin fibroblasts and epithelium.*—Fragments of foreskin were explanted in the same manner as the adult muscle. These cultures were trypsinized and explanted on glass in 20 percent calf serum and 80 percent Eagle's medium. The cells were tested on the 16th day *in vitro*.

4) *Normal human fetal muscle.*—Small fragments of muscle from a 4½-month fetus were explanted in T-30 flasks on glass in 20 percent human serum and Eagle's medium. Small, dense islands of spindle-shaped cells which developed over a period of 25 days were trypsinized

and explanted in a series of D-35 Carrel flasks. These cells were tested on the 29th day *in vitro*.

5) *Normal human leukocytes.*—Citrated peripheral blood was allowed to stand for 1 hour at 10°. The supernatant plasma layer was centrifuged at 1500 rpm for 10 minutes and the plasma discarded. Cells of the buffy coat were resuspended in human serums/Eagle's medium and about 3×10^5 cells were explanted in D-35 Carrel flasks and tested with antiserums 12 to 24 hours after culture.

6) *Acute lymphatic leukemia (human).*—This material was handled like normal human leukocytes after a similar dilution in human serum and Eagle's medium.

7) *Carcinoma of ovary–ascites (human).*—Thirty cc of ascitic fluid from a patient with advanced cancer of the ovary was centrifuged at 1500 rpm for 10 minutes. The clear supernatant was discarded and the layer of tumor cells and macrophages diluted with human serum/Eagle's medium and explanted in D-50 Carrel flasks. The cultures contained macrophages and small islands of easily identified tumor cells after 24 hours *in vitro*.

8) *HeLa.*—The HeLa cell line we used has been carried since 1955 in a medium composed of calf serum and medium 199. This cell line was originally sent to Dr. D. Karzon in Buffalo by Dr. J. Syverton.

9) *Rabbit ovary fibroblasts.*—This is a rapidly multiplying, permanent cell line derived from fragments of adult rabbit ovary in October, 1959.

10) *Chinese hamster kidney fibroblasts.*—This is a rapidly multiplying strain derived from fragments of adult male hamster kidney in November, 1959.

The Sillen antihuman cancer serum received from Dr. Björklund was tested against human cells and rabbit and Chinese hamster fibroblasts at 1:3 to 1:20 dilutions of the antiserums (with medium 199). When complement in the form of guinea-pig serum was added to the system, it replaced part of the medium 199 (*see* table 1).

RESULTS

The freshly reconstituted Sillen antihuman cancer serum had little cytotoxic activity toward human cells when diluted more than 1:10. At 1:20 dilution of antiserum only retraction of the cytoplasm and cell clumping occurred in the absence or presence of complement in HeLa, normal fetal, and adult muscle and foreskin fibroblasts, which were observed for 48 hours.

No obvious cell damage to either normal or cancer cells occurred before 3 hours of incubation in the presence of complement at 1:10 dilution of the antiserum. At lesser dilutions, the Sillen anticancer serum caused more rapid degenerative changes and lysis of all the human cells against which it was tested. An example of these cytolytic changes is shown in figure 2 and may be compared with the normal control cells in figure 1.

New explants of ovarian carcinoma ascites cells, as well as normal macrophages in the ascitic fluid, which might be considered as normal

TABLE 1.—The effect of Sillen anticancer serum on normal and malignant cells *in vitro**

Experimental test media and dilutions	HeLa cell from cancer of cervix (in lab. since 1955)†	Human ovarian carcinoma cells and macrophages from advanced cancer of ovary (24 hours *in vitro*)	Normal muscle fibroblasts from surgical specimen (10 and 12 days)	Amnion from human term delivery (7 and 10 days)	Normal leukocytes from normal male donor (20 hours)	Acute lymphatic leukemia from untreated patient with acute lymphoblastic leukemia (20 hours)	Rabbit ovary fibroblasts from adult New Zealand white rabbit (9 months)	Chinese hamster kidney fibroblasts from adult hamster (10 months)
1 Normal horse serum 1 Guinea-pig serum 2 Medium 199	−	−	−	−	−	−	−	−
1 Freshly reconstituted Sillen serum 3 Medium 199	+	±	+	+	+	+	−	−
1 Sillen serum heated at 56° C for 45 min 3 Medium 199	−‡	−‡	−‡	−‡			−	−
1 heated Sillen serum 1 Guinea-pig serum 2 Medium 199	+	+	+	+	+	+	−	−
1 Sillen absorbed with normal human liver 1 Guinea-pig serum 2 Medium 199	−	−	−	−	−	−	−	−
1 Sillen absorbed with normal rat liver 1 Guinea-pig serum 2 Medium 199	+	+	+	+	+	+	−	−

* + = Cell degeneration and lysis; − = no effect.

† Times are given *in vitro* before testing with normal and Sillen horse serum.

‡Slight retraction and rounding up of cells after 24 hours in antiserum medium.

cells, were also affected in a similar way. Normal white cells, macrophages, polymorphonuclear leukocytes, and leukemic leukocytes (lymphoblasts and small numbers of normal macrophages) formed clumps of cells when Sillen serum was added. Swelling of the cytoplasm was observed in cells on the periphery of these clumps. These cells came loose from the glass and floated in the medium within an hour after exposure to the antiserum.

No human cells grown in mediums containing normal horse serum showed these cytotoxic changes. Under these experimental conditions it was evident that all the human cells, whether normal or malignant, were damaged by the Sillen antiserum.

When the Sillen serum was heated to 56° for 45 minutes or stood at 5° for 72 hours, cytotoxic activity decreased, as evidenced by the fact that when diluted with medium 199 no alterations occurred in the morphology of any human cell types studied during a 4- to 5-hour observation period. HeLa, normal amnion, and normal muscle fibroblast cultures, which were incubated for 24 hours with the heat-inactivated serum, showed slight retraction and clumping of cells, but no marked cell granulation, blistering, or lysis. However, rapid degeneration and lysis occurred when complement was added to the heated Sillen serum similarly diluted with medium 199 (*see* table 1).

Absorption of the Sillen serum with sediments of normal human liver prepared in our laboratory removed the cytotoxicity for all the human cells, whether of normal or malignant origin. The same serum absorbed with rat liver sediment (equivalent to the wet weight of human liver used) retained its cytotoxic activity for all human cells. The Sillen serum did not produce any cytotoxic changes in rabbit or Chinese hamster cells before or after absorption with human or rat liver sediments. Guinea-pig complement enhanced the cytotoxic effect of the absorbed serum. A summary of the effects of the normal and Sillen horse serums on representative normal and malignant cell types is shown in table 1.

The pooled, lyophilized cancer tissue received from Dr. Björklund completely neutralized the cytotoxicity of the Sillen serum for both (cancer) HeLa and normal fetal muscle fibroblasts. These cultures were closely observed for 48 hours and during this time no cytotoxic changes occurred. When the Sillen serum was absorbed with human lyophilized liver, there was a definite delay of 4 to 5 hours before cytotoxic changes were observed in HeLa cells and normal fetal muscle fibroblasts. After 12 hours both types of cells developed marked degenerative changes (blistering of cytoplasm, granulation, and pyknosis). All HeLa cells and normal fetal fibroblasts in these cultures were lysed after 24 hours. Sillen serum absorbed with lyophilized human brain or adult skeletal muscle was as cytotoxic as nonabsorbed serum. The cytotoxic effects were observed within 2 hours and complete degeneration of the HeLa and fetal fibroblast cultures occurred in 10 hours of incubation (*see* table 2).

Because of these interesting findings with Björklund's lyophilized tissue preparation, a normal tissue (umbilical cord) containing primarily stromal

TABLE 2.—Cytotoxicity after absorption of Sillen anticancer serum

Absorbing material	Experimental mediums	HeLa	Normal human fetal fibroblasts
Equal parts of fresh, pooled human plasma and Sillen serum	1 Sillen serum Human plasma 5 Eagle's medium	Marked cell damage and lysis Cell alterations after 2 hours	Marked cell damage and lysis Cell alterations after 2 hours
Cancer*	1 Sillen serum 1 Guinea-pig serum 5 Eagle's medium	Observed for 48 hours No cell damage	Observed for 48 hours No cell damage
Liver*	1 Sillen serum 1 Guinea-pig serum 5 Eagle's medium	Cell alterations after 5 hours Complete degeneration at 24 hours	Cell alterations after 5 hours Complete degeneration at 24 hours
Skeletal muscle*	1 Sillen serum 1 Guinea-pig serum 5 Eagle's medium	Cell alterations at 2 hours Marked cell damage and lysis at 5 hours, with few cells on glass	Cell alterations at 2 hours Marked cell damage and lysis at 5 hours, with few cells on glass
Brain*	1 Sillen serum 1 Guinea-pig serum 5 Eagle's medium	Cell alterations at 2 hours Marked cell damage and lysis at 5 hours, with few cells on glass	Cell alterations at 2 hours Marked cell damage and lysis at 5 hours, with few cells on glass

*Each of these tissues which represents 100 mg of lyophilized, pooled human tissue (sent to Roswell Park Memorial Institute by Dr. Bertil Björklund) was absorbed with 1.2 cc of Sillen serum (*note* "Methods").

elements was used to absorb the Sillen serum in the same manner as the other lyophilized tissues. When this absorbed serum was tested on normal foreskin fibroblast and HeLa cells at the same dilutions (*see* table 2), no cytotoxicity could be demonstrated in the absence or presence of added complement. Again the nonabsorbed Sillen serum was toxic for the normal foreskin fibroblasts and HeLa cells. It appeared that the connective tissues which comprised the human umbilical cord contained many or all the antigens responsible for the cytotoxicity toward normal and malignant cells. This confirmed our previous observations concerning the localization of anti-HeLa rabbit serum in human stromal tissues (*13*).

HeLa and fetal muscle fibroblasts treated with Sillen serum absorbed with pooled human plasma were both lysed as readily as cells exposed to nonabsorbed Sillen serum and there was no delay of cytolysis, which indicated that no cytotoxic antibodies were removed by plasma. Similar results were obtained when lyophilized, pooled human plasma was used to absorb the Sillen serum. After absorption, the Sillen serum was toxic for HeLa cells, normal foreskin fibroblasts, and epithelium. Sillen serum absorbed with human plasma and then heated at 56° for 45 minutes caused only cell retraction and clumping when tested on the normal and malignant cells. Normal horse serum absorbed with the human plasma had no effect on the normal or malignant cells.

DISCUSSION

In earlier publications Björklund reported that a crude particulate sediment, which was obtained from normal rabbit bone marrow after ball-mill grinding and discarding of an ethyl-ether soluble fraction, produced complement-fixing antiserums when injected into horses. The antiserums were capable of causing cell damage *in vivo* and eventually death when injected intravenously in rabbits. Normal horse serum did not cause these effects (*19*, *20*).

Pools of a variety of human carcinomas were prepared in the way described for the earlier work with normal rabbit marrow, and were used to immunize a horse, "Sillen." According to Björklund, "The tumor pool was not homogeneous. It contained connective tissues, different types of reticulo-endothelial cells, epithelial cells in various stages of differentiation, and also small amounts of other specific cells belonging to the tissues from which the tumors were taken. . . . Under such circumstances it could be expected that the antigen analysis should reveal antigens which are shared by many different tissues," (*16*, *17*, *21*) and such was the case. As in earlier work with rabbit bone marrow, the antigenic analysis could be used only for soluble antigens, and the number of antibodies directed against particulate, normal, or abnormal cellular antigens could not be analyzed.

It is known that in some antigen-antibody systems complement is not fixed. Horse antibodies, though unable to fix guinea-pig complement when they are directed against pneumococcal capsular polysaccharide antigen, are able to do so against pneumococcal protein antigen (*22*). The presence in the horse of all components of complement capable of reacting with other antibody antigen systems (*23*) indicated that the horse carries its own lytic system intact.

There is a striking similarity between the activity of horse serums of antinormal rabbit bone marrow and horse antihuman cancer (*19*, *20*, *24*). Hematological changes have occurred in patients treated with Björklund's antihuman cancer horse serum absorbed with normal human plasma which are very similar to the changes seen in rabbits treated with anti-rabbit bone marrow horse serum. In both rabbits and patients there was a thrombocytopenia, leukocytosis, and drop in hemoglobin. Although patients have died due to acute glomerular nephritis (3 of 14 patients), their clinical pictures were analogous to serum sickness. There was no way to control these studies and the possible effect of antinormal tissue activity superimposed on serum sickness cannot be separated easily (*24*).

The presence of cytolytic antibody to normal cellular components is self-evident in light of the crude antigen preparations used, yet in none of his publications does Björklund report the presence of these contaminating species-specific antibodies in his cancer-specific antiserum. Moreover, the cytotoxic antibodies to normal cellular components were not demonstrated even though the antiserum was used in high concentration and complement was present (*16*). Ample evidence for normal cellular antibodies can be seen, however, from the gel-diffusion studies (*21*). How were these species-specific cytolytic antibodies missed in Björklund's experiments and how can plasma absorptions alone remove or inactivate them?

Since rabbit antiserum to human globulin (*14*), human fibrinogen (*25*), or tissue-culture mediums did not lyse the various human cells in culture even in the presence of complement, one is led to believe that the removal of the antibodies not directed at various cell types, *i.e.*, horse antiplasma activity, might not be as important here as the use of tissue absorptions.

Our general experience with explants of tumor tissues has been that tumor cells may occasionally survive and show some outgrowth together with normal connective-tissue elements when fragments are explanted in plasma clots; often only the normal parenchymal or stromal elements survive *in vitro* and they show an active rate of outgrowth and multiplication. In the experiments reported by Björklund (*16*, *17*), when tumor tissue was explanted on agar blocks or in plasma clots only tumor cells in these cultures were damaged.

In our experiments the normal and malignant cells were grown under the same tissue-culture conditions before they were exposed to the test serums. All cells were explanted on glass in Carrel flasks, on slides, and in T-30 flasks and bathed in a fluid medium so that when Sillen antiserum was added an intimate contact was immediately made between

the test serums and all cells. Plasma clots were not used because they obscured detailed microscopic observation of cells and interfered with degeneration and lysis. Björklund, on the other hand, explanted cells on agar blocks, in chicken plasma clots, and on glass. He, when dealing with cytopathogenic effects of immune bodies, noted that the clot might constitute an important variable. However, when the explanted cells were exposed to antihuman cancer serum diluted in mediums containing 40 percent human serum, cancer cells degenerated while normal cells were not affected.

Our absorption experiments with Björklund's lyophilized cancer tissue confirmed his observation that all toxicity was removed from the Sillen serum for HeLa cells. However, cytotoxicity was also removed for normal cells in culture after absorption with the lyophilized cancer tissues. This indicated that the antigens responsible for production of cytotoxic antibodies for both normal and malignant cells were present in high concentrations in the pool of cancer tissue. Similar antigenic substances must be present in normal umbilical cord since the Sillen serum absorbed with lyophilized umbilical cord lost all its cytotoxicity. If there was a specific cancer antigen in all the cancer tissues used to immunize the horse Sillen, one would expect that the specific cytotoxic cancer antibody to human cancer cells would remain in the serum after absorption with umbilical cord.

Absorption of the antiserum with lyophilized human liver caused a definite reduction in the cytotoxic reaction toward normal and malignant cells, which indicated the presence of smaller quantities of the antigens responsible for cytotoxicity than in cancer pool or umbilical cord. The skeletal muscle and brain apparently contained no antigens responsible for cytotoxicity. The fact that under our experimental conditions it was possible to demonstrate a reduction in cytotoxicity after absorption with Björklund's human liver preparation indicated that our method of assaying cytotoxicity *in vitro* was more sensitive.

Cell alterations occur rapidly when cytotoxic antiserums are properly diluted in the presence of complement. Under these conditions, marked degeneration and detachment of cells was observed. Severe cytotoxic alterations were noted within 30 minutes to 5 hours with 1:3 to 1:10 dilutions of the Sillen serum. At greater dilutions of antiserum, proportionately longer incubation was required before any visible cytotoxic effects occurred and these involved only cell retraction and clumping. In the absence of guinea-pig serums (complement) there were no marked degenerative cell alterations. The effect (species-specific) was visible on all human cell types used here, both normal and malignant. The effect was not visible on permanent strains of Chinese hamster or rabbit cells.

Under our experimental conditions Björklund's anticancer serum prepared in a horse against a pool of human cancer tissue after absorption with pooled human plasma was cytotoxic for both normal and malignant human cells growing *in vitro*. Björklund's results (*16*, *17*) indicate,

however, that after plasma absorption the serum can specifically kill only tumor cells and not normal cells.

The cancer cell and the normal cell from which it is derived share many protein and other antigenic substances. It is a well-established fact that there are certain antigenic modifications in neoplastic cells when they are compared with the normal species, isoantigens and tissue antigens (*26–29*). These antigenic modifications are most frequently exhibited as antigenic weakening or loss, but include instances of the appearance of new antigens (*30*). However, the new antigenic substances are not generally cancer-specific and usually not cross-reactive with similar substances in other tumors. They are heavily overshadowed in their reactivity by persisting normal antigens characteristic of the species or the tissue which would act as targets of cytotoxic antibody and mask more subtle differences between normal and neoplastic cells. Absorption of Björklund's antihuman cancer serum with normal human tissue or plasma does not reveal a residue of cancer-specific antibody directed against an antigen characteristic of neoplasia as such.

REFERENCES

(*1*) LUMSDEN, T.: Tumour immunity. Effects of eu- and pseudo globulin fractions of anti-cancer sera on tissue cultures. J. Path. & Bact. 34: 349–355, 1931.

(*2*) ———: Tumour immunity. Am. J. Cancer 15: 563–640, 1931.

(*3*) LAMBERT, R. A., and HANES, F. M.: The cultivation of tissues *in vitro* as a method for the study of cytotoxins. J. Exper. Med. 14: 453–461, 1911.

(*4*) LAMBERT, R. A.: A note on the specificity of cytotoxins. J. Exper. Med. 19: 277–282, 1914.

(*5*) PYBUS, F. C., and WHITEHEAD, H. R.: Immune reactions and cancer. J. Path. & Bact. 32: 195–202, 1929.

(*6*) NIVEN, J. S. F.: Action of cytotoxic antiserum on tissue cultures. J. Path. & Bact. 32: 527–550, 1929.

(*7*) PHELPS, H. J.: Note on non-specific action of so-called "anti-cancer serum." Am. J. Cancer 31: 441–445, 1937.

(*8*) MOUNTAIN, I. M.: Cytopathogenic effect of antiserum to human malignant epithelial cells (strain HeLa) on HeLa cell culture. J. Immunol. 75: 478–484, 1955.

(*9*) MILLER, D. G., and HSU, T. C.: The action of cytotoxic antisera on the HeLa strain of human carcinoma. Cancer Res. 16: 306–312, 1956.

(*10*) MCALLISTER, R. M., GRUNMEIER, P. W., CORIELL, L. L., and MARSHAK, R. R.: The effects of heterologous immune serums upon HeLa cells *in vitro* and rat-HeLa tumors *in vivo*. J. Nat. Cancer Inst. 21: 541–555, 1958.

(*11*) GOLDSTEIN, G., and MYRVIK, Q. N.: The reversible and irreversible toxic effects of anti-HeLa cell rabbit serum upon human cell lines in tissue culture. J. Immunol. 80: 100–105, 1958.

(*12*) GOLDSTEIN, M. N.: The cross reactions of antisera prepared against four human cell strains in continuous tissue culture. J. Immunol. 79: 113–117, 1957.

(*13*) HIRAMOTO, R., GOLDSTEIN, M. N., and PRESSMAN, D.: Reactions of antisera prepared against HeLa cells and normal fetal liver cells with adult human tissues. Cancer Res. 18: 668–669, 1958.

(*14*) GOLDSTEIN, M. N., HIRAMOTO, R., and PRESSMAN, D.: Comparative fluorescein labeling and cytotoxicity studies with human cell strains, HeLa, Raos, and 407-liver and with fresh surgical specimens of cervical carcinoma, osteogenic sarcoma, and normal adult liver. J. Nat. Cancer Inst. 22: 697–705, 1959.

(*15*) HIRAMOTO, R., GOLDSTEIN, M. N., and PRESSMAN, D.: Limited fixation of antibody by viable cells. J. Nat. Cancer Inst. 24: 255–265, 1960.

(*16*) BJÖRKLUND, B., GRAHAM, J. B., and GRAHAM, R. M.: Effect of horse anti-human cancer serum on malignant and normal human cells. Internat. Arch. Allergy 10: 56–64, 1957.

(*17*) BJÖRKLUND, B., and BJÖRKLUND, V.: Antigenicity of pooled human malignant and normal tissues by cyto-immunological technique; presence of an insoluble, heat-labile tumor antigen. Internat. Arch. Allergy 10: 153–184, 1957.

(*18*) BJÖRKLUND, B., LUNDBLAD, G., and BJÖRKLUND, V.: Antigenicity of pooled human malignant and normal tissues by cyto-immunological technique: II. Nature of tumor antigen. Internat. Arch. Allergy 12: 241–261, 1958.

(*19*) BJÖRKLUND, B., and HELLSTRÖM, L.: Studies on the effect of anti-bone-marrow serum. Acta med. scandinav. 139: 122–132, 1951.

(*20*) BJÖRKLUND, B.: Immunologic studies of rabbit bone-marrow with the aid of gel diffusion and serologic techniques. Internat. Arch. Allergy 4: 340–359, 1953.

(*21*) ———: Antigenicity of malignant and normal human tissues by gel diffusion techniques. Internat. Arch. Allergy 8: 179–192, 1956.

(*22*) KABAT, E. A., and MAYER, M. M.: Experimental Immunochemistry. Springfield, Ill., C C Thomas, 1948, p. 130.

(*23*) LANDY, M., MICHAEL, J., TRAPANI, R. J., ACHINSTEIN, B., WOODS, M. W., and SHEAR, M. J.: An antibody-complement system in normal serum lethal to mouse tumor cells. Cancer Res. 20: 1279–1291, 1960.

(*24*) DE LA PAVA, S., NIGOGOSIAN, G., and PICKREN, J. W.: Fatal acute glomerular nephritis following administration of a horse anti-human cancer serum. Arch. Int. Med. In press.

(*25*) GOLDSTEIN, M. N., HIRAMOTO, R., and DAY, E.: Unpublished observations.

(*26*) HAUSCHKA, T. S., and AMOS, D. B.: Cytogenetic aspects of compatability. Ann. New York Acad. Sc. 69: 561–577, 1957.

(*27*) WEILER, E.: Antigenic differences between normal hamster kidney and stilboestrol induced kidney carcinoma complement fixation reactions with cytoplasmic particles. Brit. J. Cancer 10: 553–559, 1956.

(*28*) O'GORMAN, P., and MIKULSKA, Z. B.: The antigenic structure of a transplanted sarcoma. Brit. J. Cancer 14: 121–125, 1960.

(*29*) GORER, P. A.: Interactions between sessile and humoral antibodies in homograft reactions. *In* Cellular Aspects of Immunity. Boston, Little Brown & Co., 1960, pp. 330–347.

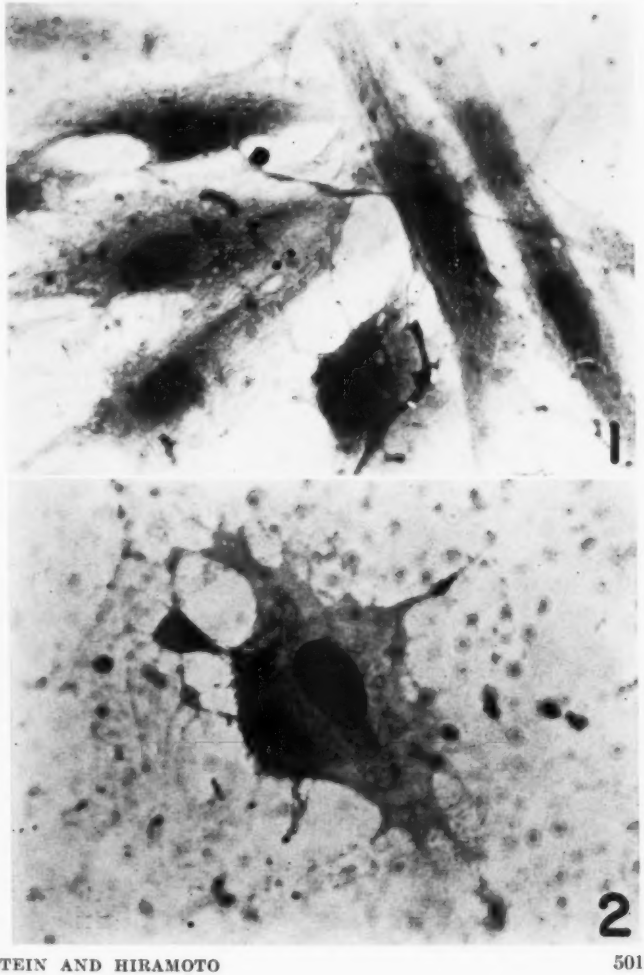
(*30*) GORER, P. A., and AMOS, D. B.: Passive immunity in mice against C57BL leukosis E. L. 4 by means of isoimmune serum. Cancer Res. 16: 338–343, 1956.

PLATE

PLATE 57

FIGURE 1.—Normal human muscle fibroblasts growing on glass in fluid medium for 12 days. Lillie fixation. Hematoxylin and eosin. × 500

FIGURE 2.—Culture similar to that of figure 1: 12 days *in vitro*. Exposed to Sillen antihuman cancer serum and guinea-pig complement—1:4 dilution for 30 minutes. Most of the cells were lysed and only debris remained on glass. Cytoplasm of this cell is swollen and cell membrane distorted. Numerous blisters on cell surface. Lillie fixation. Hematoxylin and eosin. × 600



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Experimental Mammary Adenocarcinoma of Rats: Some Considerations of Methylcholanthrene Dosage and Hormonal Treatment.[1,2]

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SUMMARY

Huggins *et al.* (J. Exper. Med. 109: 25-42, 1959) shortened the time required for induction of mammary carcinoma by modifying dosage and rat strain in a system that was originally devised in this laboratory. We confirmed this effect for the Wistar strain rat also. There appears, however, to be an optimal level to which this dose may be increased for any particular strain of rat beyond which toxic effects of the carcinogen rapidly supervene. For the Sprague-Dawley rat, this limit approximates a 10 mg daily dose. Our results indicate that in the Wistar strain a 10 mg daily dose of 3-methylcholanthrene intragastrically is exceedingly toxic and that the 5 mg dose is ideally suited to this strain of rat. Two parameters for evaluation of neoplasia should be considered in this system: 1) incidence that appears to depend, within limits, on the total dose of carcinogen administered; 2) mean appearance time of the first tumor dependent, within limits, on the size of the daily dose of methylcholanthrene. The optimal *total* dose of 3-methylcholanthrene for the Wistar rat is approximately 300 mg. There is a minimal latent period, about 2 months for the Wistar rat, that is independent of the size of either daily or total dose of methylcholanthrene. The percentage of animals that developed tumors (incidence) and mean time of palpable appearance of first tumors may each be influenced independently by the hormonal environment.—J. Nat. Cancer Inst. 27: 503-513, 1961.

THE APPEARANCE of a mammary adenocarcinoma after the gastric instillation of methylcholanthrene in female rats was first described by Shay *et al.* (*1*). The relation of the sex hormones to the incidence of this mammary neoplasm after the gastric instillation of methylcholanthrene has been reported from this laboratory (*2*) and confirmed by Huggins *et al.* (*3*) and Dao and Sunderland (*4*). Further studies from this laboratory have added to these observations and extended them to related steroidal compounds (5, *6*). The purpose of this communication is to elaborate on the experimental reproducibility of this system, to define

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more closely the roles of the sex hormones as therapeutic agents against this neoplasm, and to examine the effects of large daily doses of methylcholanthrene on breast-cancer induction in female rats. The Wistar rat strain has been used by us exclusively during the past 10 years to study the relation of endocrine factors to the induction of this tumor.

MATERIALS AND METHODS

Groups of 20 or more noninbred 6- to 8-week-old female Wistar rats, grown in our colony, were selected by statistical randomization. Each animal was housed individually and received a daily dose of 2 mg, 5 mg, or 10 mg of methylcholanthrene by stomach tube (7) in 0.5 cc olive oil 6 times per week for varying periods. All animals were observed for 12 months and final evaluation was made at that time. Twenty-five mg pellets of estradiol and/or 75 mg pellets of testosterone were implanted either singly in the right lateral subcutaneous fold or in juxtaposition when both hormones were used.

All animals were maintained on a Rockland diet and tap water *ad libitum*. When tumors appeared, they were palpated weekly and removed when they exceeded 2 cm in maximum diameter.

The percentage of animals that developed tumors (incidence) was determined at the end of 52 weeks, and the mean time of palpable appearance of first tumors (MAT) was computed for each group. The χ^2 method (by the use of Yates' correction factor) served to compare incidence between groups, while the Student t test was used to determine significance between differences in the time of onset of first tumors.[4]

RESULTS

Methylcholanthrene Treatment

The daily gastric instillation of 2 mg of 3-methylcholanthrene for 12 months increased the mean incidence of mammary adenocarcinoma from zero in untreated females to 81 percent of 85 animals treated (table 1). Moreover, this mean incidence of mammary carcinoma after 12 months of methylcholanthrene treatment (table 1) significantly exceeded that noted after 4 months of similar therapy (58% of 152 rats, table 2). Although incidence was increased by prolonging methylcholanthrene treatment, the mean times of appearance of first tumors were not significantly different (27.0 vs. 28.7 weeks, tables 1 and 2).

The reproducibility of breast-cancer induction with a daily 2 mg dose of methylcholanthrene is demonstrated in these tables. Daily administration of 2 mg of methylcholanthrene for as short a period as 1 month

[4] χ^2 and t values for experimental groups are compared against suitable control groups or, in the case of reproducibility studies, against the mean of the individual values.

TABLE 1.—Reproducibility of mammary adenocarcinoma by daily gastric instillation of 2 mg of methylcholanthrene (12 months)*

Group No.	Number of rats			Incidence (percent with tumors)	Weeks to appearance of first tumor (MAT) ± SD
	Total	Developed tumors	No tumors		
282	18	12	6	67	28.9 ± 8.3
282.1	29	22	7	76	33.5 ± 10.7
360	21	17	4	81	27.6 ± 9.8
301.8	17	17	0	100	26.0 ± 9.3
Total	85	68	17		
Mean ± SE				81 ± 7.0	28.7 ± 1.64

*Animals not treated on Sundays.

provoked a marked rise in the final incidence of mammary adenocarcinoma (31% of 16 rats treated, table 3). Further dosage for an additional 2 months doubled the incidence (67% of 15 rats treated), and maximal incidence was achieved after approximately 6 months of therapy (88% of 16 rats treated, table 3).

An increase in daily dose to 5 mg or 10 mg produced a maximal incidence with a 2-month treatment period. Furthermore, an increase in the *daily carcinogen dose*, within limits, decreased MAT. It is important to note, however, that MAT was independent of *duration of carcinogen therapy* (total dose, table 3) but related to the *daily* carcinogen dosage used.

Effects of Estrogenic Hormone Alteration

Ovariectomy, panhysterectomy, or implantation of a 25 mg pellet of estradiol after a 4-month daily regimen of 2 mg of methylcholanthrene reduced tumor incidence by about 50 percent, and each therapeutic measure significantly delayed appearance of the first tumors (table 4). Estradiol implantation *per se* was associated with only 1 tumor in 24 rats

TABLE 2.—Reproducibility of mammary adenocarcinoma by daily gastric instillation of 2 mg of methylcholanthrene (4 months)*

Group No.	Number of rats			Incidence (percent with tumors)	Weeks to appearance of first tumor (MAT) ± SD
	Total	Developed tumors	No tumors		
295	20	9	11	45	27.5 ± 11.7
295.1	25	16	9	64	29.8 ± 10.3
301.4	16	10	6	63	26.3 ± 13.2
308.5	21	12	9	57	30.3 ± 12.1
356	20	10	10	50	26.1 ± 5.4
417	36	25	11	69	23.9 ± 10.6
425	30	17	13	57	25.0 ± 10.6
Total	168	99	69		
Mean ± SE				58 ± 3.1	27.0 ± 0.9

*Animals not treated on Sundays.

TABLE 3.—Effect of dosage and duration of therapy on incidence and time of onset of mammary adenocarcinoma*

Group No.	Duration of administration (months)	Cumulative dose (mg)	Developed tumors	No tumors	Incidence (percent with tumors)	Weeks to appearance of first tumor (MAT ± SD)
2 mg Methylcholanthrene						
301.1	1	54	5	11	31	31.8 ± 11.2
301.2	2	108	5	10	33	25.2 ± 16.9
301.3	3	162	10	5	67	22.9 ± 11.8
301.4	4	216	10	6	63	26.3 ± 13.2
301.5	5	270	11	5	69	33.3 ± 11.3
301.6	6	324	14	2	88	27.3 ± 9.7
301.7	9	486	14	2	88	27.1 ± 10.7
301.8	12	648	17	0	100	26.0 ± 9.3
4 mg Methylcholanthrene						
388	12	1296	19	2	91	21.4 ± 8.6
5 mg Methylcholanthrene						
439	1	135	12	8	60	17.3 ± 5.8
436	2	270	20	2	91	16.5 ± 5.1
437	3	405	19	1	95	15.8 ± 3.3
440	4	540	17	4	81	17.1 ± 4.0
441	12	1620	20	1	95	20.9 ± 7.5
10 mg Methylcholanthrene						
394	1	270	14	9	61	17.4 ± 5.2
394.1	2	540	16	4	80	19.9 ± 9.7
394.2	3	810	15	6	71	16.2 ± 5.5
394.3	4	1080	15	3	84	17.5 ± 7.3

*Animals not treated on Sundays.

treated, and the time of appearance of this tumor was similar to that which occurred with methylcholanthrene induction. When estradiol (25 mg pellet) was implanted at the start of methylcholanthrene treatment and continued to the end of the experiment (12 months), tumor incidence was reduced approximately 50 percent, but MAT was not changed appreciably (table 4).

Effects of Androgenic Hormone Alteration

By contrast, testosterone-pellet implantation and simultaneous methylcholanthrene administration for 12 months reduced tumor incidence from that noted with methylcholanthrene alone (81 vs. 33%, table 5). Moreover, MAT was delayed from 28.7 to 39.2 weeks ($P = <0.05$).

Implantation of testosterone after 4 months of methylcholanthrene treatment did not significantly alter the *incidence* of mammary carcinoma from that of methylcholanthrene-treated rats but did increase MAT significantly ($P = <0.05$). The most striking prophylactic effect in reducing tumor incidence was noted when testosterone therapy and ovariectomy were used after 4 months of methylcholanthrene treatment (table

TABLE 4.—Effect of estrogenic alteration on incidence and time of onset of mammary adenocarcinoma induced by daily administration of 2 mg methylcholanthrene*

Group No.	Treatment	Number of months	Number of rats	Developed tumors	No tumors	Incidence (percent with tumors)	χ^2	Weeks to appearance of first tumor (MAT) ± SD	t Value
Control	Methylcholanthrene (MC)	4	152	89	63	58		27.1	
321	MC, then oophorectomy	4	22	6	16	27	<0.05	34.5 ± 8.5	<0.10
377	MC, then panhysterectomy	4	23	6	17	26	<0.05	39.3 ± 13.3	<0.05
298	MC, then estradiol pellet implanted	4	21	3	18	28	<0.05	38.0 ± 10.2	<0.10
298.1			22	9	13		N.S.		<0.001
			43	12	31				
278	MC, estradiol pellet implanted at start of MC treatment & continued × 12 months	4	17	5	12	29	<0.05	30.0 ± 4.9	N.S.
Control	MC	12	85	68	17	81		28.7 ± 9.5	
435	Estradiol	12	24	1	23	4	<0.001	30.0 ± 0	N.S.
361	MC, estradiol pellet implanted × 12 months	12	21	8	13	38	<0.05	29.8 ± 7.4	N.S.

*Animals not treated on Sundays.

TABLE 5.—Effect of testosterone on incidence and time of onset of mammary adenocarcinoma after daily administration of methylcholanthrene*

Group No.	Treatment	Number of months	Number of rats	Developed tumors	No tumors	Incidence (percent with tumors)	χ^2	Weeks to appearance of first tumor (MAT) ± SD	*t* Value
Control	Methylcholanthrene	4	168	99	69	58		27.1	
297	Testosterone administered after methylcholanthrene	4	19	8	11	42	N.S.	33.4 ± 9.8	<0.05
322	Testosterone & ovariectomy after methylcholanthrene	4	23	2	21	9	<0.01	40.0 ± 12.8	N.S.
403	Testosterone, estradiol & ovariectomy after methylcholanthrene	4	33	13	20	39	<0.05	36.9 ± 13.5	<0.05
Control	Methylcholanthrene	12	85	68	17	81		28.7 ± 9.5	
307	Testosterone administered with methylcholanthrene	12	31	10	21	32	<0.05	39.2 ± 2.45	<0.05

*Animals not treated on Sundays.

5). In 2 of 21 treated rats that developed mammary adenocarcinoma, MAT was delayed until 40 weeks. Combination therapy, comprising ovariectomy, testosterone, and estradiol after 4 months of methylcholanthrene administration (table 5) was no less effective than combined ovariectomy and testosterone therapy in delaying MAT, but *incidence* of tumors was more than trebeled when the estradiol was added (table 3). The action of testosterone appears to have converted the response to the 25 mg estrogen pellet to a small dose effect. Thus, tumor incidence was markedly increased when androgenic and estrogenic therapy were combined with ovariectomy as compared to the result with the addition of testosterone alone.

DISCUSSION

The reproducibility of the induction of mammary adenocarcinoma after administration of 3-methylcholanthrene intragastrically to female Wistar rats has been reported earlier (*8*). Reconsideration of these data and more recent results have led us to believe that 2 parameters should be considered in quantitating the results of this method: first, incidence, *i.e.*, the percentage of animals that develop breast cancer; second, the time elapsed from the beginning of carcinogen administration to the palpable appearance of the first tumor (MAT).

It was anticipated that a correlation would exist between these 2 parameters, but such was not the case. Thus (table 3), while an increase in the daily dose of methylcholanthrene decreased the mean appearance time of the first tumor as first pointed out by Huggins *et al.* (*3*), final incidence was related rather to the total dose of carcinogen administered. While increasing the daily dose of carcinogen above 2 mg decreases MAT, this effect becomes limited in the Wistar rat at a daily dose of 5 to 10 mg of carcinogen. Thus 10 mg of methylcholanthrene given daily did not decrease MAT beyond that produced by the 5 mg dose (table 3). Furthermore, there exists a latent period of approximately 2 months, and this period is independent of both the daily and the total dose of methylcholanthrene used in these experiments. Since 2 months is the minimum time required for tumor development after carcinogen therapy is started in this strain, and since MAT is a function of the daily carcinogen dose, an increase in the daily dose of methylcholanthrene can only decrease appearance time to a functional limit of 2 months.

The relationship of incidence to the total dose of carcinogen is illustrated in table 3 by the increase in the percentage of animals finally developing breast cancer. These results were 31 percent after 1 month of administration of 2 mg daily, 67 percent after 3 months of administration, and 88 percent after a 6-month period of treatment. After larger total dosage (5 or 10 mg daily dose), no benefit to incidence appeared to accrue beyond the optimally effective *total* dose which in the Wistar rat appears to be about 300 mg (table 3). Also indicated in table 3 is the relatively greater toxicity of the 10 mg daily dose in this strain. Thus incidence was ad-

versely affected by the 10 mg dose compared with that of the 5 mg dose, while MAT was not decreased further by the larger dose. This increased toxicity was also indicated by a relatively high early death rate in the 10 mg group, which did not occur with either the 2 or 5 mg dose. Huggins *et al.* (*3*) using the Sprague-Dawley strain and a daily methylcholanthrene dose of 10 mg reported a 100 percent incidence of breast cancer in 109 rats within 22 to 77 days. When the dose was increased to 15 mg, tumors appeared in only 24 of 72 rats in 34 to 69 days, the other 48 rats having died without tumors between 34 and 69 days after the experiment was begun. Thus the optimal daily dose of methylcholanthrene used in this system must be determined for each strain of rat employed.

When we administered a 2 mg daily dose of methylcholanthrene for different periods, we found that the administration of the carcinogen in this dose induced breast cancer after 4 months of treatment in approximately 20 percent of female rats. If administration of methylcholanthrene was stopped at this time and the female rats which had not yet developed breast cancer were set aside, approximately half of them subsequently developed breast cancer in the ensuing 6 to 8 months (table 2). We therefore consider those rats without tumors at the end of 4 months of methylcholanthrene administration as groups of animals, in approximately half of which a pattern for carcinogenesis had been laid down. Prophylaxis in these animals must be reconciled with the possibility that some tumors becoming palpable shortly after cessation of methylcholanthrene treatment might represent growth of a microscopic tumor that was already present at the end of the 4-month period of carcinogen administration. Such studies have been reported (*9*).

Since the latent period for appearance of breast cancer after administration of any of the daily doses used was not less than 2 months, and since 2 months of administration of a 5 mg daily dose of methylcholanthrene in the Wistar rat induces tumors in approximately 90 percent of the treated animals, this schedule was best suited to this strain of rat when prophylactic measures against this tumor were to be investigated.

That the 2 parameters, incidence and MAT, should be considered when the results of this system of breast-cancer induction are equated is emphasized by the dichotomous response to therapy that may result. Ovariectomy or subcutaneous estradiol implantation after 4 months of methylcholanthrene administration decreased incidence approximately 50 percent and significantly increased MAT. However, when methylcholanthrene and estradiol therapy were continued simultaneously for the full 12 months, only *incidence* of tumors was reduced, whereas MAT remained unchanged (table 4). In the female the effects of estradiol implantation when combined with methylcholanthrene seem inconsistent, since an increase in breast cancer in the male has been noted with this combination of treatment (*8*). Similarly, the effects of ovariectomy in the female under similar conditions mitigates breast-cancer induction so that incidence of breast cancer was reduced from 89 to 19 percent; additionally, the mean appearance time of the first tumor was more than

doubled. Huggins *et al.* have confirmed these results with ovariectomy as well as the effects of large doses of estrogen in the female and have attributed the paradoxical results to a "biphasic effect of estrogens" (*10*). These investigators found that small amounts of phenolic estrogens returned the incidence of breast cancer induced by 10 mg daily of methylcholanthrene in ovariectomized rats to that of the *unspayed* female, whereas higher estrogen dosage inhibited tumor induction. Huggins *et al.* felt that "lactational proliferation" inhibits or delays mammary carcinogenesis. Experimental mammary neoplasia has been found to regress during lactation (*11, 12*), but these occurrences are relatively inconsistent; indeed the reverse occurs clinically. By contrast, the effects of ovariectomy or large doses of estrogen are remarkably consistent. A thesis favoring decreased incidence with lactational proliferation in this system should be reappraised after a consideration of the effects observed after testosterone pellet implantation in methylcholanthrene-treated female rats (table 5). In these studies, the dose of testosterone was such that no lobulo-alveolar or ductal hyperplasia was observed; indeed diminution in total glandular tissue within the mammary tree has been noted (*13*). Moreover, this hypothesis has been refuted in this system with the Sprague-Dawley rat (*4*).

When testosterone was implanted for the 12-month period of methylcholanthrene administration, a significant decrease in incidence and an increase in MAT were noted (table 5). However, identical testosterone therapy used 4 months after methylcholanthrene administration provided no significant decrease in incidence, but MAT was delayed ($p = <0.05$). Testosterone implantation combined with ovariectomy after 4 months of methylcholanthrene administration protected all but 2 of the 23 rats in this group from developing breast cancer (decreased incidence). MAT in these animals (group 322, table 5) was 40 weeks compared with 27.1 weeks for methylcholanthrene-treated animals. The difference, while biologically clear, could not be statistically evaluated because so few animals contributed to this mean time of tumor appearance. The addition of estradiol therapy to the regimen of testosterone and oophorectomy increased incidence more than threefold (from 9% in group 322 to 39% in group 403, table 5) but did not significantly alter MAT (40 weeks and 36.9 weeks, table 5). Estradiol, when implanted after 4 months of methylcholanthrene therapy, reduced incidence from 58 to 28 percent ($p = <0.05$) and also delayed the mean appearance time of first tumors from 27.1 to 38 weeks.

The physiological potency of estradiol per unit weight is greater by at least 100-fold than that of testosterone. It is therefore possible that, with the combination of testosterone and estradiol in a ratio of only 3 : 1, the more potent of these materials (estradiol) will prevail to influence the environment of the host and impart its own type of carcinogenic pattern. Moreover, although no testosterone pellets were found at necropsy, which indicated complete absorption and perhaps a waning androgenic effect, estradiol pellets were always present. In this case it

appears that the large dose of estradiol (25 mg) was partially counteracted by the testosterone imposed (group 403, table 5). Thus, the biological imposition of a small dose effect of phenolic estrogen increased the incidence of breast cancer over that observed with testosterone and ovariectomy alone (group 322, table 5).

These results support the postulate (*8*) we suggested several years ago to explain the inhibitory effect of large doses of phenolic estrogen on the incidence of methylcholanthrene-induced breast cancers in our system. Rats given high doses of estradiol invariably develop large chromophobe adenomas. Thus, at the end of a year of observation, a group of 19 untreated female rats had a mean pituitary weight of 5.57 mg per 100 g body weight. Two groups of animals given methylcholanthrene (2 mg daily dose for 4 months) showed mean pituitary weights of 5.49 and 5.56 mg per 100 g body weight, respectively. Two groups of 12 and 14 rats treated with the same daily dose of carcinogen for 1 year showed respective pituitary weights of 5.51 mg and 5.49 mg per 100 g body weight. On the other hand, 2 groups of 8 and 6 rats that received only pellets of 25 mg of estradiol implanted subcutaneously at 60 to 80 g of weight and killed a year later showed mean pituitary weights of 136.70 mg and 140.10 mg per 100 g body weight, respectively. Similar pituitary enlargement was noted when the estradiol pellet was combined with methylcholanthrene, and the degree of enlargement proved to be directly related to the length of time estradiol was effective. Thus when methylcholanthrene was administered for 4 months and an estradiol pellet was implanted at the end of this time, pituitaries with mean weights in 14 and 12 female rats of 69.24 mg and 74.98 mg per 100 g body weight, respectively, resulted during the succeeding 8 months. Estradiol pellets implanted for a year, with methylcholanthrene administered for the same period, produced a mean pituitary weight of 117.38 mg per 100 g body weight.

All groups receiving methylcholanthrene with large doses of estradiol showed a marked suppression of breast-cancer incidence, despite the *low* grade carcinogenic action of estradiol *per se* on the breast. We therefore postulated that large doses of estradiol inhibited a cocarcinogenic factor of pituitary origin whose action was to enhance methylcholanthrene induction of mammary cancer in the rat. When an estradiol pellet is added to ovariectomy and testosterone pellet implantation, the increased incidence (group 403, table 5) over that observed with only ovariectomy and testosterone treatment (group 322) lends credence to this premise. The mean pituitary weight in group 403 (table 5) of 22.48 mg per 100 g body weight represents a marked reversal of the effect of estradiol on the pituitary by implantation of testosterone, which thus allows the "pituitary factor" to contribute to the increased incidence of breast cancer in this group.

REFERENCES

(1) SHAY, H., AEGERTER, E. A., GRUENSTEIN, M., and KOMAROV, S. A.: Development of adenocarcinoma of the breast in the Wistar rat following the gastric instillation of methylcholanthrene. J. Nat. Cancer Inst. 10: 255–266, 1949.

(2) SHAY, H., HARRIS, C., and GRUENSTEIN, M.: Influence of sex hormones on the incidence and form of tumors produced in male or female rats by gastric instillation of methylcholanthrene. J. Nat. Cancer Inst. 13: 307–331, 1952.

(3) HUGGINS, C., BRIZIARELLI, G., and SUTTON, H.: Rapid induction of mammary carcinoma in the rat and the influence of hormones on the tumors. J. Exper. Med. 109: 25–42, 1959.

(4) DAO, T. L., and SUNDERLAND, H.: Mammary carcinogenesis by 3-methylcholanthrene. I. Hormonal aspects in tumor induction and growth. J. Nat. Cancer Inst. 23: 567–585, 1959.

(5) SHAY, H., HARRIS, C., and GRUENSTEIN, M.: Further studies in prevention of experimentally induced breast cancer in the rat. Some endocrine aspects. Acta Unio internat. contra cancrum 16: 225–231, 1960.

(6) ———: Studies in prophylaxis of methylcholanthrene induced breast cancer in the rat. Proc. II Internat. Symp. on Mammary Cancer, University of Perugia, July 24–29, 1957, 753–764.

(7) SHAY, H., and GRUENSTEIN, M.: A simple and safe method for the gastric instillation of fluids in the rat. J. Lab. & Clin. Med. 31: 1384–1386, 1946.

(8) SHAY, H., GRUENSTEIN, M., and HARRIS, C.: Reproducibility of method and hormonal influences in induction of breast cancer in rats by gastric instillation of methylcholanthrene. Acta Unio internat. contra cancrum 12: 733–741, 1956.

(9) HARRIS, C., SHAY, H., GRUENSTEIN, M., and CIMINARA, J. L.: Breast tumor formation as a response to graded doses of methylcholanthrene and the effect of this drug on the pituitary glands of rats. Acta Unio internat. contra cancrum 15: 595–692, 1959.

(10) HUGGINS, C., GRAND, L. C., and BRILLANTES, F. P.: Critical significance of breast structure in the induction of mammary cancer in the rat. Proc. Nat. Acad. Sc. 45: 1294–1300, 1959.

(11) BIELSCHOWSKY, F.: The carcinogenic action of 2-acetylaminofluorene and related compounds. Brit. M. Bull. 4: 382–385, 1947.

(12) HADDOW, A.: The biological characters of spontaneous tumours of the mouse, with special reference to rate of growth. J. Path. & Bact. 47: 553–565, 1938.

(13) SHAY, H., HARRIS, C., and GRUENSTEIN, M.: Progress Report to National Institutes of Health—Grant CY-2590 (C4), October, 1959.

Mammalian Chromosomes *In Vitro*. XV. Patterns of Transformation [1, 2]

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SUMMARY

The chromosome constitution of a number of cell strains derived from mouse tissues was studied in detail. Strains H_{1a}, H_{1b}, H_{1c}, and H_{4a} were heteroploid (with chromosome numbers in the subtetraploid range) when the first samples were taken. At that time they were 9 to 11 months of age *in vitro*. Strain H_{4c}, initiated and sampled at the same time as strain H_{4a}, was primarily diploid. Strain H_2 was principally diploid 5 months after its primary cultivation. In its control line, H_{2c}, tetraploid, and later subtetraploid, cells gradually gained dominance and became the stemline. In populations of strain H_2, treated intermittently with 5-bromodeoxyuridine, polyploidization was accelerated in one line (H_{2a}). In another line, H_{2b}, a temporary reversion to near diploid (s =38) was noted. However, cells in the subtetraploid region finally became the stemline also. Thus heteroploid transformation may undergo the following 2 patterns: diploidy-tetraploidy-hypotetraploidy and diploidy-subdiploidy-hypotetraploidy. Structural changes of chromosomes were constantly observed in all cell lines, especially those with heteroploid stemline. Most biarmed chromosomes had equal arms, suggestive of isochromosome formation. However, few, if any, metacentrics were incorporated into the stemlines of the strains analyzed. The thymidine analogue, 5-bromodeoxyuridine, induces chromosome breakages and accelerates polyploidization.—J. Nat. Cancer Inst. 27: 515–541, 1961.

AFTER REPEATED reports of contamination of cells in tissue-culture lines (*1–4*), a number of investigators began to doubt whether there is a process of heteroploid transformation or alteration *in vitro* except under highly special circumstances. Actually, strain L cells of the mouse, the main culprit of cellular contamination, are themselves products of transformation, for their chromosomal patterns are entirely different from normal mouse karyotype (*1*, *2*) yet antigenically are definitely of murine origin. Cell strains in the studies of Chu, Sanford, and Earle (*5*) represent

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an independent set of altered strains, because their chromosome constitutions resemble neither those of strain L nor those of the normal mouse.

Levan and Biesele (*6*), using a cell strain derived from embryonic mouse skin, studied its chromosome cytology from the primary culture until a total transformation was reached. In their laboratory, then in the Memorial Sloan-Kettering Cancer Center, New York, no strain L was carried. Similarly, Hsu and Klatt (*7*) studied 3 strains of the Novikoff rat hepatoma and found that all of them changed their chromosomal composition after growth *in vitro* for approximately 10 months. Several marker chromosomes of that tumor proved beyond doubt the identity of these cells.

In both cases of transformation mentioned, a pattern was observed: the predominance of diploid or near-diploid cells in the initial culture and early period of culture, with a shift toward tetraploidy as cultivation continued. New karyotypes, above and below the tetraploid level, might constantly be generated, and at one stage the populations might contain numerous karyotypes so that no distinct peak or peaks could be seen in the distribution curve of chromosome number classes. Later, certain heteroploid stemlines, usually with chromosome numbers below those of the double original stemline, gradually increased in proportion and finally the mode settled at a heteroploid number or several connecting numbers.

It is important to ascertain that heteroploid transformation often takes place in cell cultures, and it is also of interest to determine whether transformation invariably follows a single pattern. In cultivation of bone marrow of the mouse, Billen and Debrunner (*8*) described a culture procedure whereby cell strains were established from primary cultures in nearly 100 percent of attempts. This finding offered an opportunity for studies of morphological, cytological, physiological, and biochemical changes during various stages of strain development.

In the present report, chromosomal data, including numerical and structural variations collected from several mouse cell strains, are presented to illustrate the transformation process *in vitro*.

MATERIALS AND METHODS

Strains H_1 and H_4 were initiated from bone marrow of C3H mice, the former on January 27, 1959, and the latter, August 13, 1959. The history of these lines, the medium constituents, and other pertinent technical data have been presented previously (*8*). Originally a number of primary cultures was set up for each strain. As individual cultures grew, they were propagated without being mixed with any other culture. Thus each initial culture, when established, represented a separate strain. Three such lines of H_1, designated as H_{1a}, H_{1b}, and H_{1c}, and 2 of H_4, designated as H_{4a} and H_{4c}, were analyzed cytologically.

Strain H_2 was initiated in one of our laboratories on June 29, 1959,

from a piece of peritoneum of a C3H female. It was cultivated in NCTC 109 medium with 10 percent horse serum until August 4, 1959, when a change was made to NCTC 109 with 20 percent fetal calf serum.

RESULTS

Somatic Chromosomes of the Mouse

It is well known that the diploid number of the mouse is 40, all of which are telocentric. Their sizes range roughly from 2 to 6 μ and they do not form distinct groups according to length. Therefore only a few of the longest and the shortest chromosomes can be identified. In some mouse strains, 1 chromosome pair with a characteristic proximal heterochromatic differentiation, affectionately termed "the rabbit-ear chromosome," is recognizable. Figure 1, showing diploid cells from strain H_{2c}, may represent the normal mouse karyotype. Figure 13 illustrates the normal chromosomes of an embryonic cell (C3H) from a primary culture aligned in the order of decreasing size. The rabbit-ear chromosomes are #13 and 14 from the right. Detailed data will be published (*9*) on the chromosomes of strains C3H, C57BL, and Swiss.

Strain H_1

The first set of samples of the 3 H_1 lines for cytological studies was taken 11 to 12 months after the initial culture. All the populations examined had already become heteroploid. Two additional sets of chromosomal characterization were made for H_{1a}, and 1 additional set for H_{1b}. Table 1 presents the results of observations collected from these populations.

Among the 3 samples from line H_{1a}, the first had a relatively wide spectrum and a low peak (stemline?) at 74 and 75. The second and third samples, taken 9 days apart, were apparently similar in composition. The stemline number was 66 to 67, and the spectrum was narrower than that of the first. A similar situation existed in the 2 samples of H_{1b} in which the first sample contained a wide spectrum of chromosome number classes with a low peak at 64, and the second showed a narrowed spectrum without a change in the stemline number. In the only sample of line H_{1e} taken about the same time as the first samples of the other 2 lines, the population was already totally heteroploid with its stemline number at 70 to 71 chromosomes.

One karyotype analysis each of lines H_{1a} and H_{1b} is shown in figures 14 and 15, respectively. It is evident from these figures that the size range of the chromosomes in the cell lines exceeds that of the normal mouse cells, which suggests cryptostructural variation during the development of the strains in addition to the numerical changes. The presence of minute chromosomes was striking at all 3 samplings of H_{1a}. In a slide from the third sample of H_{1a}, 55 of the 62 cells studied possessed from 1 to 6 minutes

TABLE 1.—Distribution of chromosome number classes in populations of strain H_1; initial cultures set on January 27, 1959

Cell line	Sample date	40	41	42	49	53	58	59	60	61	62	63	64	65	66	67	68	69	70
H_{1a}	12/15/59			1					1			2	1	3		2			2
	5/10/60								1			4	4	4	8	7	6	3	4
	5/19/60						1				1	1	2	2	3	12	11	9	7
H_{1b}	1/8/60				1	1	1	3	2	2	1	4	7	5	3	4	5	3	3
	5/19/60	1	1	1					2	2	2	4	6	2	1	1	1		
H_{1c}	1/2/60												1	2	2	2	2	5	11

71	72	73	74	75	76	77	78	80	82	88	90	>100	Cells studied	s*	M*	min*
2	2	2	6	5	1	3		1			1		35	74	9	2
1		1	2	1					1	1		5	53	66	–	–
3	1	1					2					6	62	67	10	55
1	1		1		1								50	64	11	15
			1									6	31	64	10	11
12	6	3	2	1	1								50	71	36	6

*s= Stemline chromosomes number: M = number of cells with metacentric chromosome(s); min = number of cells with minute chromosome(s).

(average 2.3 minutes per cell). These minutes were often very small (fig. 14). Many of them were morphologically indistinguishable and might actually represent the same minute in varying numbers. They often attached with their centromeric end to one of the larger chromosomes (fig. 32) in a manner similar to the behavior of chromosome 21 of man, which often shows terminal association with chromosomes 13 to 15.

Indications of structural variation other than the formation of minutes were seen in every sample of all cell lines. One instance from H_{1a} is depicted in figure 39, in which some chromosomes of a cell with double stemline number (133) are illustrated, including 1 dicentric, 2 large acentrics, and some tiny paired centric or acentric fragments. In another instance, also from H_{1a}, a large number of chromosome breaks had taken place in 1 cell. An analysis of this cell gave a total of 138 separate chromosome elements, including 48 monocentric chromosomes of ordinary type, 8 centric minutes, 4 dicentrics, 4 metacentrics, 67 acentrics, 4 acentric minutes, and 2 acentric rings. The total number of centromeres was 68, close to the modal number which was 67. The number of acentric chromosomes indicates the absolute minimum number of breaks that must have taken place. Few other cells from the same culture showed more than 3 breaks per cell.

It is of special interest that in all these lines, though cells possessing all telocentric chromosomes were common, biarmed chromosomes were not infrequent (table 1). A number of cells contained 1 or 2 metacentrics, but some contained 4 or 5 such elements. Instances of metacentric chromosomes from strain H_{1a} are pictured in figures 25 to 32, most of which also show the largest and/or the smallest chromosomes of the cell for comparison. It is evident that the biarmed chromosomes varied in size, which indicated that they were not descendants of a single or a few anomalies. A survey of 31 metacentrics, chosen at random and drawn by camera lucida at a magnification of 2500, revealed that the two arms of most of the metacentrics were equal in length (text-fig. 1; fig. 26). It must be emphasized that sometimes, as illustrated in figures 30 and 32, the biarmed chromosomes may in reality represent terminal associations of 1 large and 1 small telocentric. Careful examination, however, would enable one to differentiate the *bona fide* metacentrics from terminal associations.

Strain H_4

As described by Billen and Debrunner (*8*), strain H_4 was initiated from mouse bone marrow in McCoy's 5a medium containing no glucose other than that present in the fetal calf serum. The cultures grew much slower than those in medium containing glucose. One sample was taken from a population of line H_{4a} approximately 10 months after its initiation. In this population no diploid elements were encountered. Most cells were in the subtetraploid range with a stemline number at 75 (table 2). One karyotype of 77 chromosomes is shown in figure 16; this karyotype has characteristics similar to those of strain H_1.

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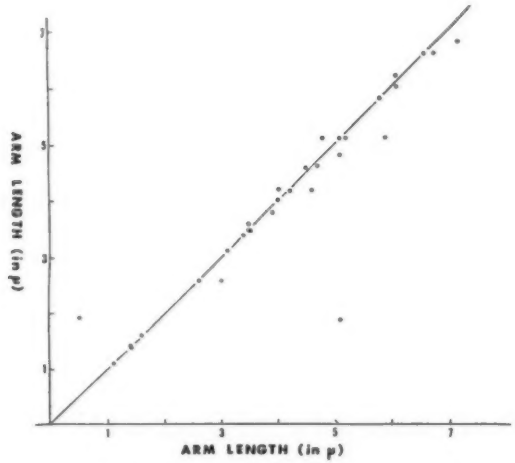


TEXT-FIGURE 1.—Distribution of arm lengths among 31 biarmed chromosomes found in cells of strain H_1.

TABLE 2.—Distribution of chromosome number classes in populations of strain H_4; initial cultures set on August 13, 1959

Cell line	Sample date	39	40	41	42	71	72	73	74	75
H_{4a}	5/19/60	–	–	–	–	1	1	1	3	10
H_{4c}	4/19/60	–	23	1	–					1
	5/19/60	6	27	4	4					1

76	77	78	79	80	81	82	160	Cells studied	s*	Percent in diploid zone†
6	5	2	–	1	–	1	–	31	75	0
				1			2	28	40	80.0
1	1	5	7	10	2			68	80	37.5

* s = Stemline chromosome number.
† Data from 200 or more cells read at random.

The chromosome composition of line H_{4c} was quite different from that of H_{4a}, though both strains were initiated at the same time. The first sample of H_{4c}, taken approximately 9 months after the primary culture, showed almost exclusively diploid elements. Only 2 aneuploid cells were recorded from a sample of 28 cells. Among the 3 polyploid cells critically examined, 1 was a tetraploid (80 chromosomes) and the other 2 were octoploid (160 chromosomes).

In an attempt to test whether the karyotype with 40 chromosomes in strain H_{4c} was morphologically identical with the normal mouse karyotype, the chromosomes of 10 cells were drawn and measured individually at a magnification of 4000. The absolute measurements, as well as relative values, were compared with similar data from normal embryonic C3H

cells (*9*). The result was that the 2 groups of measurements practically coincided, with no indication of structural changes.

The similarity in chromosome length between the 2 groups of cells did not necessarily prove, of course, that no alteration whatever had occurred in the chromosomes of strain H_{4c} while *in vitro* for 9 months. Suggestions of morphological changes in the genome of H_{4c} were apparent. For example, the "rabbit-ear chromosomes" were generally less prominent than normal, and the smallest chromosome pair often had a more pronounced proximal constriction. Other constrictions usually not observed in normal mouse idiogram were relatively frequent. A karyotype, extreme in this respect, is illustrated in figure 17; almost every chromosome possesses 1 or 2 constrictions. Either this indicates a change in genotype or represents phenotypic response of the normal mouse chromosomes to an unusual environment, which is comparable to the phenomenon of "nucleic acid starvation" frequently observed in plant chromosomes. This may be induced by a number of conditions, such as cold treatment, haploidy, hybridization, and inbreeding.

One specific deviant, representing at least one structural change, was observed 6 times in 1 slide of H_{4c} among some 200 "normal" metaphases. This was a karyotype with 1 chromosome distinctly smaller than the smallest normal mouse chromosome (fig. 18). Since the minute chromosome appeared to be the same in all 6 cells, it is possible that these cells represented a karyotype, which at that moment happened to be of selective advantage and was therefore spreading in the population.

The second sample of H_{4c}, taken exactly 1 month after the first, showed a dramatic change in karyotype constitution. The majority of cells now belonged to the tetraploid region, and aneuploid variation was common at both the diploid and the tetraploid levels (table 2). The near-diploid karyotypes were carefully analyzed to detect whether the normal mouse karyotype was still predominant. Even though most cells maintained the normal karyotype, deviations were now common. Two such deviants, both with a minute chromosome, 1 with 40 and the other with 42 chromosomes, are shown in figures 19 and 20, respectively. At that time strain H_{4c} had just entered a stage at which the stemline equilibrium changed in favor of the tetraploid fraction of the population. While genotypic variation was continuing at the diploid level, the advantages gained, if any, were apparently not sufficient to compete with the variation produced in the cells at the tetraploid zone, from which the new stemline later emerged.

As in the other strains, evidence of chromosome breakage was often noticed. A structural interchange between 2 chromosomes of different sizes is pictured in figure 40.

Strain H_2

Strain H_2 was initiated from fragments of peritoneum of a C3H mouse on June 29, 1959. The first sample for cytological preparation was taken on December 1, 1959, approximately 5 months after the initial culture.

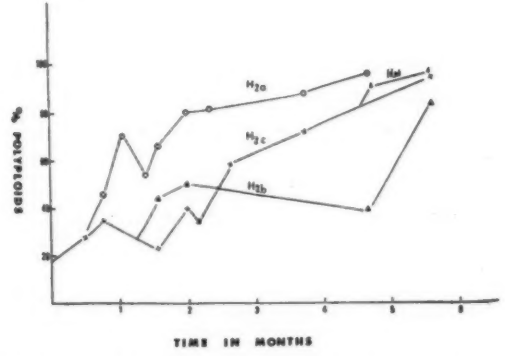
This line, designated as H_{2c}, has been kept in NCTC 109 medium with 20 percent fetal calf serum.

On December 17, 1959, 1 culture of H_{2c} was treated with 5-bromodeoxyuridine (BUDR) at a concentration of 5 μg/ml for 3 days. It was returned to the original medium for 4 to 7 days at which time a subculture was made. One subculture was fixed for cytological analysis, and the other re-treated with BUDR in a manner similar to that of the first. This subline, H_{2a}, was treated with BUDR for a total of 14 times by repetition of the cycle. The BUDR treatment had no gross effect on mitotic activity of the cells. Perhaps the presence of 10 μg/ml of thymidine in NCTC 109 was mainly responsible for allowing what appeared to be a normal growth rate to continue.

On January 11, 1960, another culture of H_{2c} was treated with BUDR. This became subline H_{2b}, which received BUDR treatment for a total of 11 times in the way just described.

On April 11, 1960, a third culture of H_{2c} was treated with BUDR twice. This subline was designated as H_{2d}.

To estimate more accurately the proportion between cells in the diploid and polyploid zone, the slides for each sample were first read only to record those 2 classes and without detailed chromosomal analysis. This method enabled us to register almost every metaphase figure, so that we eliminated bias due to the discard of unfavorable metaphase plates. Each sample consisted of a minimum of 200 cells. Text-figure 2 shows the percentage of polyploid cells (tetraploid and higher) in the 4 sublines studied chronologically.



TEXT-FIGURE 2.—Percentage of polyploids in populations of strain H_2 after treatment with 5-bromodeoxyuridine. H_{2c}, control subline. H_{2a}, H_{2b}, and H_{2d}, sublines treated with BUDR. Initial treatment: culture 5 months of age.

Text-figure 2 indicates that the H_2 population was mainly diploid at the beginning of the BUDR experiment, which was actually 5 months after the initiation of the cell strain. In the untreated control, H_{2c}, the polyploid fraction started increasing, at first hesitatingly, then rapidly and steadily, and reached 95 percent at approximately 10 months after

primary cultivation. It is obvious that the polyploidization was accelerated by the BUDR treatment in H_{2a} and H_{2b}. In the latter subline, however, there was a temporary return to diploid predominance toward the end of the 9th month.

As we just mentioned, the parental population of all the H_2 sublines contained preponderantly diploid cells (81.4%) on December 1, 1959. Even on February 2, 1960, strain H_{2c} still held a majority of diploid cells, with the stem line euploid (40 chromosomes). In the tetraploid zone the euploid number 80 prevailed. Unfortunately, exact counts were not made between February 2 and May 19, during which period the situation changed. The tetraploids had become predominant and the stemline number was now 79.

Some photomicrographs of metaphases from the diploid zone of an early H_{2c} sample are reproduced in figures 1 through 4. They have the chromosome numbers 40, 39, 41, and 42, respectively. Most chromosomes are normal in appearance, but 1 shown in figure 4 is a minute.

Subline H_{2a}, which originated from H_{2c} with BUDR treatment, showed a comparatively earlier stemline shift toward tetraploidy than the control line, H_{2c} (text-fig. 2). Euploidy, first diploidy, then tetraploidy, prevailed during the first 4 samplings. However, 1 month after the stemline had shifted to 80, the population began to turn to heteroploidy by first reducing to a stemline of 79 chromosomes and then to 77. Nevertheless, even at the last fixation, April 19, 1960, a small fraction of near-diploids remained. A fair number of cells with 39 chromosomes was observed in 1 culture, which indicated an episodically successful variant, and at this time a vigorous stemline with 38 chromosomes was present in subline H_{2b}. Results of karyotype analyses of 1 cell from the diploid zone and another from the tetraploid zone of H_{2a} are demonstrated in figures 21 and 22, respectively.

Analysis of the chromosome numbers of H_{2b} revealed an interesting development. The diploid stemline in January, 1960, shifted to hypodiploidy. On April 19, no less than ⅓ of all cells had 38 chromosomes. One month later, however, the tretaploid fraction acquired dominance, most cells then having 75 to 77 chromosomes, but the diploid minority still maintained a hypodiploid mode. The number 39 was now most frequent among the near-diploid cells. Since only a few established cases of hypodiploid stemline in mouse-cell populations are known (*10*, *11*), it should be pointed out that the present material was of such excellent quality that every cell with subdiploid chromosome count was reliable. An abundance of ideally fixed, clearly undamaged cells with 37 to 39 chromosomes convinced us that these hypodiploid cells had good viability and had replaced the euploid elements.

The stemline number 38 is, of course, especially intriguing. Detailed karyotype analyses of 10 such cells were therefore made. By drawing and measuring each chromosome of the 10 analyzed cells at a magnification of 6000, we prepared a mean idiogram and compared it with the normal C3H idiogram. The absolute sizes of the 38 chromosomes in subline H_{2b}

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were somewhat smaller than those of the normal chromosomes. The average sizes ranged from 1.5 to 4 μ, whereas those of the control cells measured from 2 to 6 μ. Other than the reduction in size of the hypodiploid cells, the 2 idiograms (fig. 23 for H_{2b} and fig. 13 for normal C3H) exhibit an almost perfect conformity. Thus it is probable that no gross translocation or other structural change was involved in the development of the subdiploid karyotype in H_{2b}. It seems unlikely, however, that a loss of 2 entire chromosomes from the normal diploid karyotype should result in increased growth capacity. It appears more reasonable to assume that cryptostructural reshuffling of genetic materials, in addition to the decrease of chromosome number, had contributed to the vigor of the growth.

The fourth subline, H_{2d}, was principally polyploid when initiated. Its stemline number was 79 but dropped to 75 in 1 month. Once again the trend of decreasing chromosome numbers, often encountered during the genetic adaptation of tetraploid cell populations, occurred. One karyotype of H_{2d} is illustrated in figure 24.

As previously mentioned, the H_2 populations frequently exhibited some chromosome breakage. At one time there was a higher frequency of chromosome breakage in the BUDR-treated populations than in the control series (H_{2a}, table 3), but this trend did not persist. Figure 5 represents a diploid cell (40 chromosomes) from the control line, which shows 2 sets of segmental interchanges and a break on 1 chromosome. Cells with extensive breakages are illustrated in figures 9 to 12. Examples of cells with unusually long or short chromosomes are given in figures 6 and 4, respectively.

Metacentric chromosomes were seen from time to time (figs. 7, 8, and 33 through 38). However, throughout the period in which the H_2 lines were followed cytologically, cells containing metacentric elements never became frequent enough in the prevailing stemline. In agreement with the results obtained from strain H_1, most metacentrics of various sizes found in H_2 were also equal-armed. Examples of large metacentrics are shown in figures 35 and 36, and a small one is illustrated in figure 37.

DISCUSSION

The Reality of Heteroploid Transformation

The chromosome constitution of our cell lines shows that, in spite of changing from diploidy to heteroploidy, all cultures were unmistakably of murine origin. In no case was there any contamination with other established cell lines, such as strain L. This verified the report of Rothfels and Parker (*10*) who observed a large number of transformed cell lines of the mouse. The phenomenon of heteroploid transformation *in vitro*, is, therefore, real and may happen repeatedly.

Although a considerable number of heteroploid cell strains are known, up to now only in a few cases have the chromosome changes been followed

TABLE 3.—Distribution of chromosome number classes in populations of strain H_2; initial cultures set on June 29, 1959

Cell line	Sample date	34	35	36	37	38	39	40	41	42	43	46	63	65	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	Cells studied	s*	M*	min*	Structure altered
H_{2c}	12/1/59						3	44	3	1																	9					60	40	1	–*	4
(control)	12/15/59						2	41	6	1													3	1	3		5		1	1		64	40	3	8	4
	12/23/59						2	25	3																	1	2	2		1			36	40	–*	–*	–*
	1/30/60			1		1	2	33	4								1			1	1	1		1	1	1	7	1			1	57	40	1	3	7
	2/2/60					1	2	15	7										1		1			2	1	2			1			33	40	1	1	1
	5/19/60					2	1	1															6	9	6	11	7	3				46	79	2	1	–
H_{2a}	12/15/59						3	42	5						1							2	1	2	1	2	3	2				64	40	0	5	5
(initial	12/23/59			1	1		5	30	7	2	4									1		1		3	3	2	5	1				66	40	3	9	15
BUDR	1/2/60					2	2	14	6														1		3	1	3	1	2				35	80	0	2	6
treatment	1/12/60					1	4	35	7	1	1	1												1	1	5	8						65	80	0	5	4
12/7/59)	1/17/60					1		19	4	1													2	1	2	5	2			1	1	39	79	1	3	4
	1/30/60						2	10	3	1													1	4	2	3	2	1	2			31	77	0	3	6
	2/10/60						3	5	1		1											1	1	1	2		1					16	77†	0	1	2
	3/22/60					1	8	1					1								1	1	4	5	2							24	77	–	–	–
	4/19/60		1		1	1	6	6	3											1	2	4	9	14	7	4						60	77	–	–	–
H_{2b}	1/17/60						3	16	5	1															2			1				28	40	1	4	5
(initial	1/30/60	1					3	9	1					1										1			1					17	40	1	1	4
BUDR	4/19/60			1	10	55	22	25	7	6	1										3	4	7	5	2	4	3	1				156	38	–	–	–
treatment	5/18/60					9	19	10											2	4	13	18	19	18	4	1						117	76	2	8	–
1/11/60)																																				
H_{2d}	4/19/60						4	4	1																2	10	4					25	79	–	–	–
(initial	5/18/60				1	1	5	2								1	1		2	4	9	11	8	3	3		2					54	75	3	7	–
BUDR																																				
treatment																																				
4/11/60)																																				

*s = Stemline chromosome number; M = number of cells with metacentric chromosome(s); min = number of cells with minute chromosome(s); – = not recorded.

during the critical stages when transformation took place, *i.e.*, when the stemline number shifted from the normal number of the host species to a new heteroploid number. This process is usually combined with a change in general properties of the cells, sometimes suddenly, sometimes gradually. The time for the onset of heteroploid transformation is, however, unpredictable.

Even though there can be no question as to the existence of heteroploid transformation, many observations indicate that normal cells may grow for long periods *in vitro* without any appreciable karyotypic alterations. Tjio and Puck (*12*) reported that in cells from normal human tissues the same karyotype was maintained for more than 5 months of rapid growth in tissue culture. Bergman and Reitalu (personal communication) have grown human-skin cells under constant chromosome checkup for as long as 18 months without chromosomal variation. In 1959, two of us (T.C.H. and A.L.) observed a large number of cultures from individual rat embryos for up to 6 months. Although a varying degree of karyotypic aberration was noted, the stemline never deviated from the normal karyotype. Among our present materials, strain H_{4c}, after 9 months of cultivation, maintained a karyotype grossly indistinguishable from the normal mouse idiogram.

Origin of Metacentric Chromosomes

Despite the frequent occurrence of structural changes in all cell lines, the normal (telocentric) chromosome type was generally maintained. Telocentric chromosomes, longer or shorter than normal, however, were common. In certain strains, such as H_1, metacentric chromosomes were more frequent than in others, though in no case, including the mouse-skin cultures of Levan and Biesele (*6*), were large numbers of biarmed chromosomes incorporated into the stemline. In strain L and the MB strains (*13*), however, a number of metacentrics could be observed in almost every cell. Whether the discrepancy is due to the difference in the age of culture (strain L is nearly 20 years old) remains unanswered.

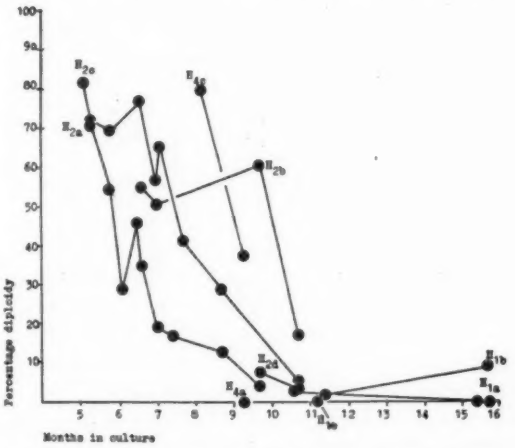
Theoretically, telocentric chromosomes may change into biarmed chromosomes by several mechanisms: 1) segmental interchange between 2 chromosomes in inverted position, with 1 chromosome breaking beyond the centromere and the other below the centromere; 2) fusion of 2 telocentrics without the loss of a centromere, which thus forms a dicentric element with the 2 centromeres that, due to their proximity, move as a single unit; 3) pericentric inversion; and 4) breakage in the centromeric region of a chromosome followed by sister chromatid fusion of the broken ends by misdivision or other mechanisms. The first 3 possible mechanisms would give rise to all gradations of asymmetrical chromosomes, while the last should yield only metacentrics. In the last category, all the metacentrics so formed should be isochromosomes. The chances of the formation of isochromosomes, originated from segmental interchange or fusion, would increase if the homologous chromosomes pair in somatic cells, as in dipterous insects. Recent reports (*14–17*) indicate that to some extent somatic pairing exists in mammalian cells, both *in vivo* and *in vitro*.

In this study, the absolute majority of the metacentrics observed had arms of equal length. Text-figure 1, in which the length of the 2 arms of each biarmed chromosome was plotted in relation to each other, emphatically suggests that most metacentrics originated as isochromosomes and not by random fusion between 2 telocentrics. In figure 7 the cell contains 40 chromosomes, 1 of which is a metacentric. It is, of course, possible that 2 separate events, a fusion between 2 telocentrics and a nondisjunction, had taken place; but it seems more probable that a misdivision of centromere had occurred on a single chromosome while the remaining elements were not involved. It must be emphasized that chromosomes with unequal arms were occasionally observed (text-fig. 1). Therefore, isochromosome formation is not the only mechanism by which biarmed chromosomes may originate in the cells *in vitro*.

Patterns of Heteroploid Transformation

In earlier investigations (*6*, *7*) a pattern was noted in the chromosomal changes of normal cells during their adaptation to tissue-culture conditions. Briefly, this pattern indicated a tendency for the original population, consisting mainly of diploid cells, to change into tetraploid, and later to heteroploid, often via a stage of spread of chromosome spectrum. We shall discuss our findings in relation to this pattern.

To obtain a picture of the disappearance of the original diploid stemline from the cultures, the percentage of diploid cells in every sample is plotted in text-figure 3. No systematic chromosome examinations were made in any culture prior to the first 5 months of cultivation *in vitro*. Some pilot observations made on early passages showed the material to be mainly diploid with some admixture of tetraploid cells. All counts were strictly euploid. We may conclude, therefore, that the chromosome status of strain H_2 at the beginning of month 5 did not deviate much from



TEXT-FIGURE 3.—The disappearance of cells in the diploid zone in various cell lines.

its original population composition, except for the slow increase of minorities of heteroploid cells around the stemline and the double stemline numbers. The heteroploid cells were not usually present in the first few passages. Thus in strain H_2, our early samplings were taken at the initial stages of the polyploidization period, which continued for 5 to 6 months, during which our analyses gave a good panorama of the course of events: tetraploidy gradually replacing diploidy, and hypotetraploidy eventually becoming predominant.

Strain H_{2b} deviated somewhat from this typical route. Polyploidization took place at first as usual, but during one period the cells in the diploid fraction again started to increase; the stemline at that time had 38 chromosomes (table 3). This is an interesting exception from the usual pattern, since it shows that near-diploid cells may occasionally compete successfully with the tetraploid fraction of the population. It is true that the dominance was short-lived, for 1 month later a stemline with 76 chromosomes took over. Whether this new stemline originated as a doubling of its successful hypodiploid predecessor or had emerged independently from the tetraploid fraction cannot be determined.

Little could be learned from strains H_1 and H_{4a} about their process of cytological changes, for in the first samples, taken at 11 months for H_1, and 9 for H_4, the populations had already concluded their heteroploid transformation. The only exception is strain H_{4c}, which was at the beginning of its polyploidization process.

Every cell line studied in this investigation eventually ended with a hypotetraploid stemline. Even strain H_{4c}, which had 80 as its most common number and thus formally had a euploid stemline, exhibited hypotetraploid variants, making 79 its average number and tending to decrease. A list of the stemline numbers for each subline taken from the last sampling is presented, arranged in the order of increasing age *in vitro:*

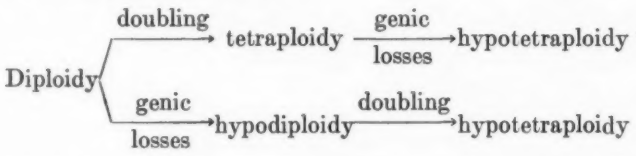
Months *in vitro*	Subline	Stemline number
8	H_{4a}	75
9	H_{4c}	80
10	H_{2a}	77
11	H_{2c}	79
11	H_{2b}	76
11	H_{2d}	75
11	H_{1c}	71
16	H_{1a}	67
16	H_{1b}	64

The material reported here is, of course, too scanty for generalizations, but a tendency of the sublines to generate a hypotetraploid stemline appeared to exist. Since the process of transformation took place over a considerable period, it is reasonable that in cell lines *in vitro* the longest should have achieved the lowest stemline number.

There is a striking similarity in chromosomal pattern between established cell strains and transplantable tumors. It is well known that many mouse tumors undergo a similar decline in stemline number during their

adaptation to an environment. Ising (*18*), in a comprehensive investigation of the behavior of tetraploid mouse ascites tumors exposed to a foreign environment, made the suggestive observation that the decrease in chromosome number was associated with actual loss of chromosome material. Thus the adaptation involves not merely rearrangements of chromosomes but also deletions.

Summarizing the discussion, we may state that available data, from this study and from elsewhere, suggest that the pattern of transformation in the mouse may follow 2 routes:



Generally the transformation process follows the first pattern. However, in both procedures the final populations would be hypotetraploid. Of course, evolution is not ended by the establishment of a certain hypotetraploid stemline. Cell strains with long history, such as strain L, undergo perpetual genetic changes and establish a large number of sublines, each with its own evolutionary pattern and genetic makeup.

Effect of 5-Bromodeoxyuridine

In microbial systems, BUDR has been known to be incorporated into cellular deoxyribonucleic acid (DNA), preferentially replacing thymine (*19–23*). In mammalian cells, this was also true (*24*, *25*). Hakala (*26*) reported that cells *in vitro*, after exposure to this agent, gradually became giant cells.

The H_2 lines treated with BUDR, *e.g.*, H_{2a}, showed during at least one period higher frequency of chromosome breakages than the control culture H_{2c}. However, the data in table 3 are not completely convincing because the populations, after repeated treatment, failed to show the remarkable difference that was apparent during the early period of treatment. The culture medium used in this study was NCTC 109, which contained thymidine. If chromosome breakages can be produced by the incorporation of BUDR, it is most likely that thymidine might have counteracted its potency. Moreover, the treatment system—3 days at a BUDR concentration of 5 μg/ml and then 4 to 7 days in normal medium—might also have lowered the BUDR effect. For these reasons, Hsu and Somers (*25*) designed experiments to test the effect of incorporation of BUDR on mammalian DNA with more favorable systems than the ones used here. It was found that BUDR not only produced extensive chromosome breakages but also produced high frequency of breakages at certain specific loci. It is safe to conclude, therefore, that the breakages observed in this series of experiments were also the result of BUDR incorporation.

Our data agree with those of Hakala (*26*) which indicate that BUDR treatment increased the proportion of giant cells (polyploidy) in cell

(12) TJIO, J. H., and PUCK, T. T.: Genetics of somatic mammalian cells. II. Chromosomal constitution of cells in tissue culture. J. Exper. Med. 108: 259–268, 1958.

(13) DE BRUYN, W. M., and HANSEN-MELANDER, E.: The chromosomes of the MB lymphosarcoma. A preliminary note. Hereditas 44: 558–559, 1958.

(14) HUNGERFORD, D. A., DONNELLY, A. J., NOWELL, P. C., and BECK, S.: The chromosome constitution of a human phenotypic intersex. Am. J. Human Genet. 11: 215–236, 1959.

(15) DURYEE, W. R., and DOHERTY, J. K.: Nuclear and cytoplasmic organoids in the living cells. Ann. New York Acad. Sc. 58: 1210–1231, 1954.

(16) RUDDLE, F. H.: Chromosome variation in cell populations. Doctorate dissertation, Univ. Calif., 1960.

(17) SANDBERG, A. A., KOEPF, G. F., GROSSWHITE, L. H., and HAUSCHKA, T. S.: The chromosome constitution of human marrow in various developmental and blood disorders. Am. J. Human Genet. 12: 231–249, 1960.

(18) ISING, U.: Effect of heterologous transplantation on chromosomes of ascites tumors: A contribution to our knowledge of environmental influence on tumor cells. Acta path. et microbiol. scandinav. Supp. 127: 1–102, 1958.

(19) ZAMENHOF, S., and GRIBOFF, G.: Incorporation of halogenated pyrimidines into the deoxyribonucleic acids of *Bacterium coli*. Nature, London 174: 306–307, 1954.

(20) ———: *Escherichia coli* containing 5-bromouracil in its deoxyribonucleic acid. Nature, London 174: 307–308, 1954.

(21) DUNN, D. B., and SMITH, J. D.: Incorporation of halogenated pyrimidines into the deoxyribonucleic acids of *Bacterium coli* and its bacteriophages. Nature, London 174: 305–306, 1954.

(22) ZAMENHOF, S. RICH, K., and DE GIOVANNI, R.: Further studies on the introduction of pyrimidines into deoxyribonucleic acid of *Escherichia coli*. J. Biol. Chem. 232: 651–657, 1958.

(23) ———: Thymine-5-bromouracil "exchange" in deoxyribonucleic acid of *Escherichia coli*. Fed. Proc. 15: 390, 1956.

(24) DJORDJEVIC, B., and SZYBALSKI, W.: Genetics of human cell lines. III. Incorporation of 5-bromo- and 5-iododeoxyuridine into the deoxyribonucleic acid of human cells and its effect on radiation sensitivity. J. Exper. Med. 112: 509–531, 1960.

(25) HSU, T. C., and SOMERS, C. E.: Effect of 5-bromodeoxyuridine on mammalian chromosomes. Proc. Nat. Acad. Sc. 47: 396–403, 1961.

(26) HAKALA, M. T.: Mode of action of 5-bromodeoxyuridine on mammalian cells in culture. J. Biol. Chem. 234: 3072–3076, 1959.

(27) HAAS, F. L., and DOUDNEY, C. O.: A relation of nucleic acid synthesis to radiation-induced mutation frequency in bacteria. Proc. Nat. Acad. Sc. 43: 871–883, 1957.

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PLATE 58

Strain H_{20}

FIGURE 1.—Diploid cell with no detectable chromosomal abnormalities; 40 telocentric chromosomes.

FIGURE 2.—Cell with 39 chromosomes.

FIGURE 3.—Cell with 41 chromosomes.

FIGURE 4.—Cell with 42 chromosomes, one of which is a minute.

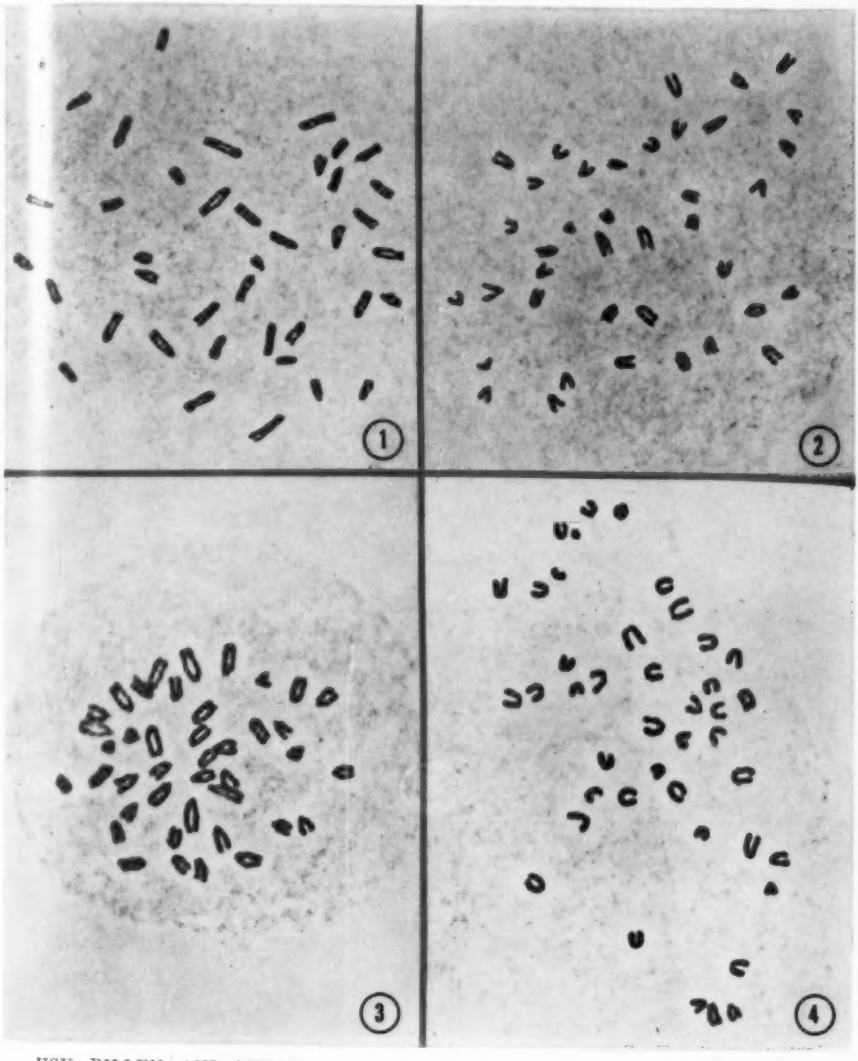
1
2
3
4

PLATE 59

Strain H_{2c}

FIGURE 5.—Cell with 40 chromosomes showing 1 broken chromosome and 2 sets of segmental interchanges. *Arrow* points to deleted centromere from lower translocation configuration.

FIGURE 6.—Cell with 38 chromosomes showing an unusually long telocentric element.

FIGURE 7.—Cell with 40 chromosomes showing a metacentric.

FIGURE 8.—Cell with 79 chromosomes showing a metacentric.

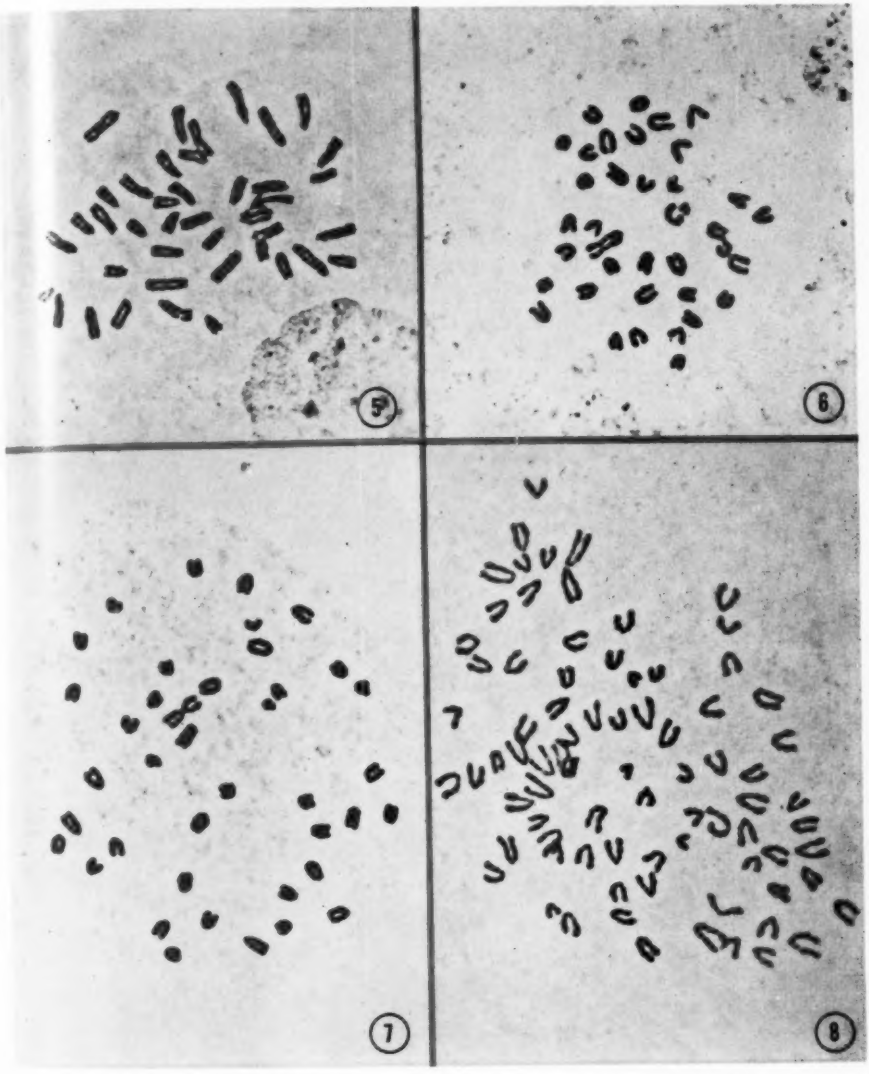

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PLATE 60

Strain H_{2a}

FIGURES 9 and 11.—Diploid cells with extensive breakages.

FIGURE 10.—High polyploid cell with extensive breakages.

FIGURE 12.—Portion of a tetraploid cell showing differential condensation of chromosomes. Also shown is an unusually long telocentric.

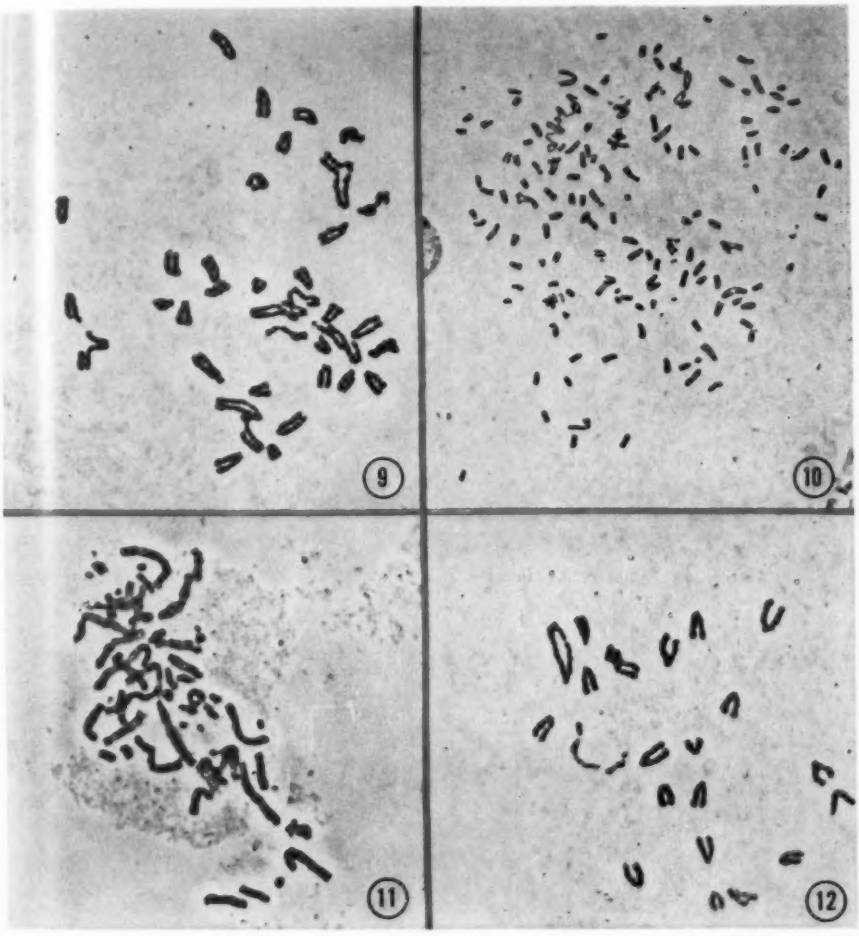

9
10
11
12

PLATE 61

Karyotypes

FIGURE 13.—Female C3H mouse, $2n = 40$ (4/25/60).

FIGURE 14.—H_{1a}, $2n = 74$ (5/10/60).

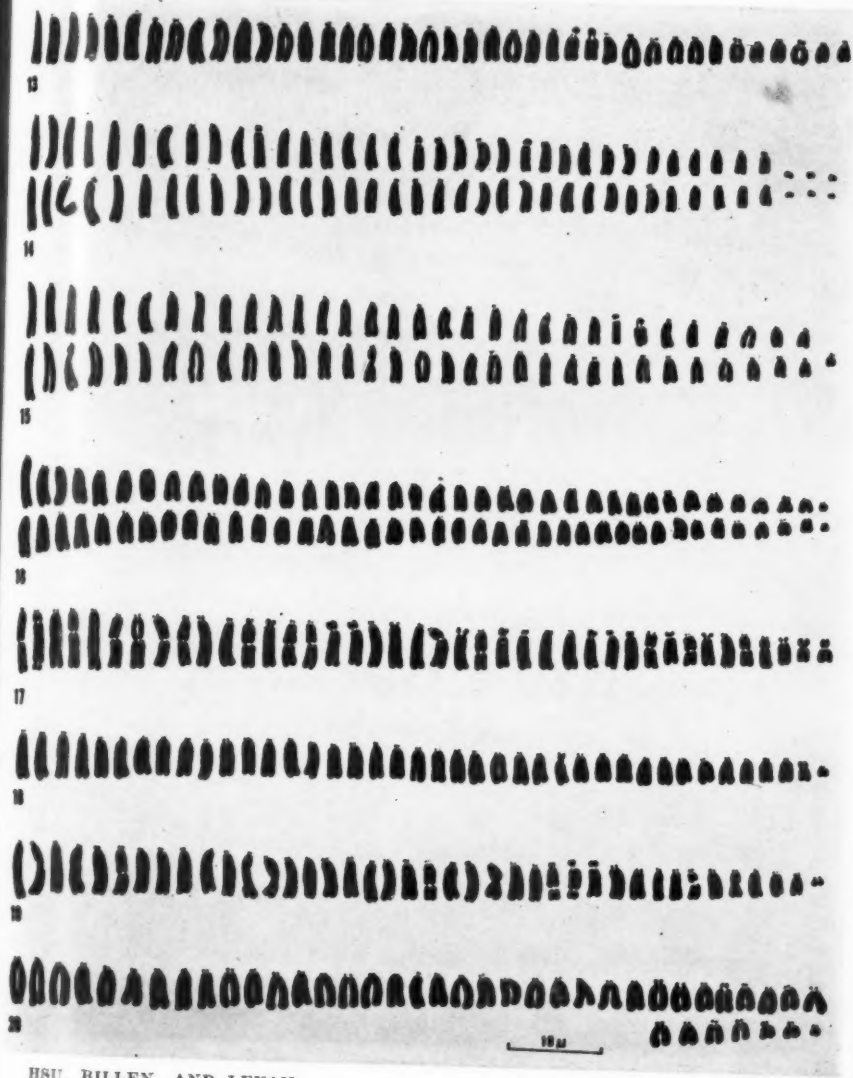
FIGURE 15.—H_{1b}, $2n = 65$ (5/19/60).

FIGURE 16.—H_{1a}, $2n = 77$ (5/19/60).

FIGURES 17 and 18.—H_{4c}, $2n = 40$ (4/19/60).

FIGURE 19.—H_{4c}, $2n = 40$ (5/19/60).

FIGURE 20.—H_{4c}, $2n = 42$ (5/19/60).


13
14
15
16
17
18
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10μ

PLATE 62

Karyotypes

FIGURE 21.—H_{2a}, $2n = 41$ (4/19/60).

FIGURE 22.—H_{2a}, $2n = 77$ (4/19/60).

FIGURE 23.—H_{2b}, $2n = 38$ (4/19/60).

FIGURE 24.—H_{2d}, $2n = 77$ (5/18/60).

Characteristic chromosomes from individual cells

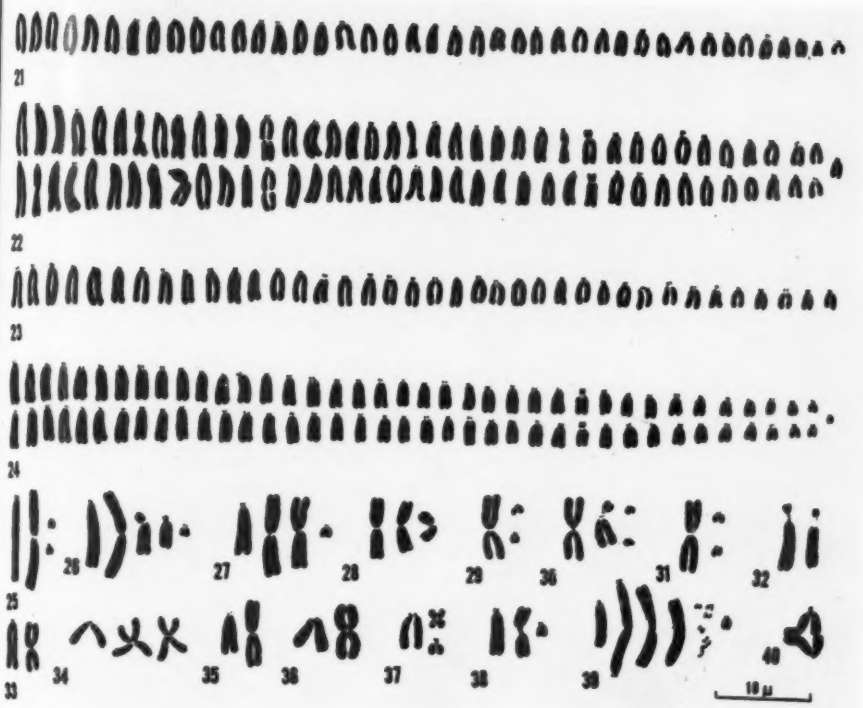
FIGURES 25 to 32, and 39.—H_{1a}.

FIGURES 33 to 34.—H_{2b}.

FIGURES 35 and 36.—H_{2c}.

FIGURES 37 and 38.—H_{2d}.

FIGURE 40.—H_{4c}.


21
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10 μ

Production of Hyaluronic Acid by Fibroblasts Growing From Explants of Walker Tumor 256: Production of Hyaluronidase by the Tumor Cells [1, 2]

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SUMMARY

In a study of the Walker tumor 256 in tissue culture, round, bipolar and multipolar, and ameboid carcinoma cells are described. When younger tumors (4 to 7 days after subcutaneous transplantation) are used for explantation, a fourth cell type appears, with small typical fibroblasts growing luxuriantly. These cells have been identified as fibroblasts of mesenchymal origin because the supernatant of such cultures formed a mucin clot which could be prevented by hyaluronidase. Since extracts of the tumor itself and supernatants of cultures from older tumors never gave a positive mucin-clot test, it has been concluded that the fibroblasts derive from the host's subcutaneous tissue which grew fibroblasts in response to tumor grafting. In older tumors, the connective-tissue barrier erected by the host's reaction was destroyed by the invading carcinoma. In fact, after 7 days, growth of this tumor was greatly accelerated. Extracts from large Walker tumors depolymerized hyaluronic acid *in vitro*. Hyaluronic acid produced in tissue culture by fibroblasts deriving from the host's subcutaneous tissue in response to tumor grafting was also depolymerized by the enzyme of the tumor extract.—J. Nat. Cancer Inst. 27: 543–558, 1961.

IN EARLY studies (*1*, *2*), invasive growth of malignant tumors was associated with spreading factors in carcinomas, and later (*3–5*) with secretion of enzymes which increase the permeability of the ground substance of the surrounding connective tissue. There was, however, no direct evidence that enzymes in a malignant tumor actually hydrolyze the mucopolysaccharides of the ground substance of the connective tissue surrounding the tumor.

Growth of the Walker tumor 256 in the animal is very slow in the first 7 days after subcutaneous transplantation. At about the 7th day an abrupt change appears to take place when growth is enormously accelerated and soon assumes large proportions. It was thought that a comparison of growth of tissue cultures prepared from this tumor, a few days

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[2] Supported by grant RG-5887, Division of General Medicine, National Institutes of Health, Public Health Service.

[3] I wish to thank Dr. J. B. Allison and Dr. W. W. Umbreit for their interest and encouragement, and Miss Betty Hauck for her valuable assistance.

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after subcutaneous transplantation, with cultures from old tumors with invasive growth would shed some light on conditions accompanying the transition from the first phase of slow tumor growth to the second phase of fast and invasive growth.

METHODS

In the present investigation a strain of Walker tumor 256 carried in Sprague-Dawley rats and transplanted subcutaneously was used for explanation.[4] In a first series of experiments the tumors were 10 to 16 days old, and in a second series younger tumors, 4 to 7 days after subcutaneous transplantation, were used. The glass floor of the culture vessels (T-60 flasks, "milk dilution bottles," and Bellco culture flasks) was coated with a thin layer of heparinized chicken plasma, just enough to make the explants stick to the glass, and 5 to 8 tissue fragments/cm^2 were explanted on the glass. After 1 to several hours at 37° C—depending on the coagulation time of the plasma—the fluid medium was added, consisting of Eagle's basal medium for monolayer cultures containing 10 percent horse serum, 10 to 20 percent half-diluted chick-embryo extract, 1 percent glutamine, and 100 μg/ml potassium penicillin and dihydrostreptomycin sulfate. The medium was renewed each 48 hours, or, for testing production of hyaluronic acid, every 72 or 96 hours. The tumors used for explantation were cleaned from central necrotic parts, and, as far as possible, from visible parts of capsular material. After 3 to 6 weeks of growth in the original culture vessels, some cultures were transferred into new vessels directly on the glass floor, either by removal of the cells from the glass floor with a rubber policeman, without the original tissue fragments, or by treatment with 0.5 percent trypsin for 5 minutes. In subsequent transfers, however, the growth rate of the replica cell cultures gradually diminished.

Highly polymerized hyaluronic acid produced *in vitro* by growing cells of mesenchymal origin has been estimated by the mucin-clot test (*6*, *7*). Addition of a few drops of 1 N acetic acid to about 1 ml sample containing highly polymerized hyaluronic acid in the presence of protein forms a tight mucin clot in a clear solution. Mucin-clot formation was prevented by the addition of 5 turbidity units of testicular hyaluronidase [5] to another sample of the substrate. The mucin-clot test is specific for hyaluronic acid present in native fluids or produced in tissue culture, and is negative for chondroitin sulfates and hyaluronic acid obtained by isolation.

The culture medium contained 20 percent half-diluted chick-embryo extract. Undiluted embryo extract prepared without saline contains 210 to 240 μg hyaluronic acid/ml and gives a tight mucin clot on acidification; 20 percent half-diluted embryo extract contains about 20 μg hyaluronic

[4] I am indebted to Dr. M. Crossley for his permission to use the facilities of his laboratory in this work, and for many discussions and suggestions.

[5] Generously supplied by Wyeth Institute for Medical Research, Marietta, Penna.

acid/ml, which is below the minimal concentration for mucin-clot production. Freshly prepared or frozen embryo extract was added to the medium. Tumor cells could be grown successfully with addition of embryo extract (*8*) as well as without (*9*). Before use, this extract gave a positive mucin-clot test. The whole medium containing 20 percent half-diluted embryo extract was again tested before use and gave no mucin clot. The amount of highly polymerized hyaluronic acid produced in tissue culture could be roughly estimated by a sample dilution method; the lowest concentration of high polymer hyaluronic acid that gives a positive mucin-clot test is about 30 μg/ml. Multiplication by 30 of the minimal amount of fluid in ml which prevents mucin-clot formation allowed a rough estimation of the concentration of hyaluronic acid in the original undiluted sample. The results agreed approximately with those obtained by turbidimetric determination.

The method of mucin-clot prevention was employed for testing hyaluronidase activity of tumor extracts (*10*). As substrate, freshly prepared embryo extract was used, or the supernatant from cultures of young Walker tumors that gave a tight mucin clot. Embryo extract as substrate for the mucin-clot prevention method was prepared as follows: 10-day-old chick embryos were put from a petri dish directly into a 30 ml syringe inserted into a large centrifuge tube, into which the embryos were squeezed. An equal volume of saline was added to the crushed embryos which were kept for 24 to 48 hours at 4° C for sufficient extraction of hyaluronic acid. After centrifugation for 20 minutes at 800 rpm the supernatant was used as substrate for mucin-clot prevention or immediately frozen for later use. Embryo extract prepared in this way gave a tight mucin clot on acidification and contained hyaluronic acid in a concentration just adequate for testing hyaluronidase activity of neoplastic and normal tissues. If fluids containing higher concentrations of hyaluronic acid, *e.g.*, synovial fluid (often containing more than 2000 μg hyaluronic acid/ml), are used as substrate, the fluid may first have to be adequately diluted for prevention of mucin-clot formation by tumor extracts usually containing low concentrations of hyaluronidase.

RESULTS

Morphology of Walker Tumor 256 in Tissue Culture

In the first series of explantations in which tumors 10 to 16 days after subcutaneous transplantation were used, the following cell forms were easily distinguishable:

1) *Large round cells, sometimes very granular with large nucleolus: nucleus and nucleus:cytoplasm ratios.*—The round cells appeared predominantly close to the explant, and, in very dense growth areas, in varying distances from the explant (fig. 1; the magnification in the figures shown in this article is approximately 230-fold). Mitoses were quite frequent. In some areas of dense growth, polygonal cells were intermingled with

round cells. Most characteristic were the prominent nucleoli, the frequent occurrence of giant and multinuclear cells, and marked variations in size of the nucleus and cells. The large round cells appeared to be the chief type of tumor cells and apparently resembled those of the mammary adenocarcinoma from which the tumor originated, though the glandular arrangement in general had been lost. When fresh fragments of the tumor were observed under coverglass in a drop of saline containing neutral red or toluidine blue (*11*), they seemed to consist only of round cells, about 50 percent of which showed normal granular vital staining.

2) *Bipolar cells, sometimes multipolar, mostly with long processes* (*figs. 2 and 3*).—These usually showed less dense growth than the round cells, and their most unusual feature was the frequent branching of the long processes. The cell body of these bipolars retained all the characteristic features of the round cells, from which the formation of the bipolars could frequently be observed directly. On one or two points of the surface of a round cell tiny protrusions appeared and gradually extended. After various periods, the otherwise unchanged body of the round cell assumed the form of a large spindle which, however, could easily be distinguished from the smaller slender, spindle-shaped fibroblast. The former was a spindle-shaped tumor cell.

3) *Variety of cell forms.*—The third cell type, which appeared as a rule after 2 weeks or more of cultivation, and, characteristically, after growth rate slowed down and distances between cells became larger, showed a variety of forms rarely encountered elsewhere. Most unusual was the frequently observed branching of long processes (figs. 6 and 7). The cytoplasm often assumed bizarre forms, but nuclear shape and the prominent nucleoli were typical for cancer cells. Abnormal mitoses, tripolar and irregular, occurred frequently. These cell forms, suggesting the emergence of a cell type able to exaggerate ameboid movement of the cytoplasm, appeared after rate of multiplication decreased and growth conditions deteriorated. In this first series of cultures from old tumors, *single* small and slender spindle-shaped cells, true fibroblasts of mesenchymal origin, might be encountered rarely, but not in larger numbers or in cell groupings.

Tests for hyaluronic acid production in the supernatants of cultures explanted from 10- to 16-day-old tumors have always been negative. Extracts prepared from the tumor itself also gave a negative mucin-clot test.

Local Reaction of the Host's Subcutaneous Tissue

In the second series of cultures in which tumors 4 to 7 days after subcutaneous inoculation were used for explantation, the same three forms of tumor cells were observed, but in addition a fourth cell type was abundantly growing—small, slender spindle-shaped cells with elongated clear nuclei and 1 to 2 nucleoli (figs. 4 and 5). Often cell strands, sheets, or long strings typical for growth of fibroblasts in tissue culture grew luxuriantly. Sometimes flattened, triangular, or multipolar mesenchymal cells were seen. Here and there, lymphocytes, single and in groups, were

intermingled with the fibroblasts. There could be no doubt of their morphological identity as fibroblasts. That they were encountered only in young tumors and were almost completely absent in older and larger tumors was evidence that these fibroblasts derive not from the tumor which originated from a mammary adenocarcinoma, but from the host; *i.e.*, the fibroblasts arise from the reaction of the subcutaneous tissue to the grafting of a carcinoma into the tissue.

Production of Hyaluronic Acid by the Host's Fibroblasts

For proof of the origin of the fibroblasts from the host's subcutaneous tissue, the supernatant from cultures was tested for hyaluronic acid. Cultures which were obtained from explantation of young tumors and which showed profuse growth of fibroblasts contained hyaluronic acid produced *in vitro* (table 1). It was most improbable that tumor cells deriving from an adenocarcinoma, an epithelial tumor, would produce hyaluronic acid *in vitro*, especially when the tumor itself did not contain hyaluronic acid as evidenced by negative tests. Only fibroblasts of mesenchymal origin produce hyaluronic acid. Such fibroblasts grow from subcutaneous tissue in response to tumor grafting. In older tumors, the barrier erected by the host's reaction is destroyed; this is why cultures from older tumors neither show growth of fibroblasts nor produce hyaluronic acid. The luxuriant growth of fibroblasts in cultures from young tumors only and the production of hyaluronic acid by those fibroblasts illustrate the local response of the host's subcutaneous tissue to tumor grafting.

TABLE 1.—Mucin-clot test in supernatants from cultures of Walker tumor 256*

Age of tumor (days)	Number of cultures	Growth	After 10 days		After 20 days		After 27 days	
			Good growth	Mucin clot+	Good growth	Mucin clot+	Good growth	Mucin clot
4–7	32	Tumor cells and profuse growth of fibroblasts	29	9	29	16	18	10
10–16	46	Tumor cells only	31	None	26	None	11	None

*The results of 14 experiments are summarized.

Production of Hyaluronidase by the Tumor Cells

Since fibroblasts producing fibers and interfibrillar substances made the local barrier against the tumor, cells of this tumor might have produced hyaluronidase, which would make possible its spread. To test this assumption, we minced freshly excised tumors to form a paste, 10 to 16 days after subcutaneous inoculation and, after adding 1 volume of saline to 2 volumes of tumor brei, we extracted them at 4° C for various periods, from 2 to 24 hours. As substrate for the mucin-clot prevention test, concentrated chick-embryo extract, freshly prepared and extracted at 4° C for 24 hours, was used, or supernatant fluid of cultures prepared from 4- to 7-day-old tumors and showing luxurious growth of fibroblasts.

Both substrates gave a tight mucin clot on acidification. Equal volumes of substrate and tumor extract were mixed to make about 1 cc, and the control contained equal volumes of substrate and saline. On addition of a few drops of 1 N acetic acid and after a vigorous shaking, a typical tight mucin clot was produced in the control tube but not in the one containing substrate with tumor extract (table 2). It has been concluded that the tumor extract contains small amounts of hyaluronidase. This is a confirmation of similar results obtained by Balazs and von Euler, who used a viscosimetric method for estimation of hyaluronidase activity (*3*).

TABLE 2.—Hyaluronidase activity of extracts of Walker tumor 256

Number of experiments	Substrate	Age of tumors	Substrate + saline	Substrate + tumor extract
3	Freshly prepared undiluted embryo extract	10–16 days after subcutaneous transplantation	Tight mucin clot	No mucin clot
4	Supernatant of cultures from 4- to 7-day-old tumors	10–16 days after subcutaneous transplantation	Tight mucin clot	No mucin clot

The prevention of mucin-clot formation by tumor extract was not due to the action of proteolytic enzymes, *i.e.*, to breakdown of the protein, which is necessary for the formation of a mucin clot; this could be seen when substrates with various concentrations of hyaluronic acid were used. Tumor extracts hydrolyzed hyaluronic acid when the substrate contained 60 to 240 μg hyaluronic acid per ml in various periods (from 5 to 20 min), and prevented mucin-clot formation. The same tumor extract did not prevent this when the substrate contained relatively high concentrations of hyaluronic acid, *e.g.*, in synovial fluid, though in all tests protein was present in approximately the same concentration.

To determine whether low molecular-weight substances such as ascorbic acid or cysteine were responsible for the depolymerization of hyaluronic acid, we dialyzed samples of the tumor extract against 0.15 N sodium chloride for various periods (*3*). No difference was observed in depolymerizing activity of dialyzed and nondialyzed samples.

Hyaluronidase Activity of Extracts From Normal Epithelial Tissues

Early reports that a spreading factor was found in carcinomas but not in sarcomas (*1*, *2*) and that extracts of a variety of normal epithelial tissues showed increased spreading led us to the idea that production of hyaluronidase by carcinoma cells may not be a quality acquired by malignant cells, but may be inherent in normal epithelial cells and utilized by carcinoma cells in an uncontrolled way. Table 3 summarizes results of experiments with tissue extracts from liver, lung, small intestine, kidney, muscle (pectoralis), subcutaneous tissue, and spleen of normal rats. Extracts from liver, lung, intestine, and kidney contained hyaluron-

dase while extracts from muscle and subcutanous tissue did not. At the same time, extracts from epithelial tissues did not contain hyaluronic acid while those from tissues of mesodermal derivation contained hyaluronic acid and gave a positive mucin-clot test (table 3). Spleen, although of mesenchymal origin, showed hyaluronidase activity and contained no hyaluronic acid. In early work on spreading factors, spleen was also found to enhance spreading (*12*).

TABLE 3.—Hyaluronidase activity of normal epithelial tissues of the rat

Tissue	Liver	Lung	Kidney	Intestine	Muscle	Subcutaneous tissue	Spleen
Mucin-clot test of tissue extract	0	0	0	0	+	+	0
Mucin-clot prevention by tissue extract added to substrate	+	+	+	+	0	0	+

Action of Hydrocortisone on Host's Response to Tumor Grafting

Since the growth of fibroblasts from young tumors only has been conceived as reaction of the host's subcutaneous tissue to tumor grafting, it was thought that hydrocortisone in high concentration, which is known to inhibit growth of fibroblasts (*13*), might therefore suppress the local reaction of the host to tumor grafting (besides other actions of this steroid). Hydrocortisone [6] in various high concentrations was thus added to the medium of 4- to 7-day-old tumors at explantation. The results in table 4 show that concentrations from 125 μg/ml upward

TABLE 4.—Action of hydrocortisone in high concentration on growth of fibroblasts from explants of 4- to 7-day-old tumors*

Concentration of hydrocortisone	Age of tumors	Growth of fibroblasts	Growth of tumor cells	After feedings without hydrocortisone
0	4–7 days after subcutaneous inoculation	Profuse growth	Very good	Very good growth
250–400 μg/ml	"	No growth	Round tumor cells dead (neutral red diffuse staining)	No growth
125–160 μg/ml	"	Growth of single cells in 20%; no growth in 80%	Tumor cells dead	Scarce growth of fibroblasts; tumor cells dead
80 μg/ml	"	Growth slightly inhibited	Good	Profuse growth of fibroblasts and tumor cells

* The results of 12 experiments are summarized.

[6] I thank Dr. R. H. Silber, Merck, Sharp & Dohme, for a generous supply of steroid hormones.

inhibit growth of both fibroblasts produced by the host in response to tumor grafting and tumor cells. However, concentration of 80 μg/ml slightly inhibits growth of fibroblasts but not of tumor cells. These findings may have some bearing on the question of the mode of action of hydrocortisone in decreasing the host's local resistance to heterologous tumor grafting, if the animal is given the compound prior to the grafting. When the tumor cells are transplanted, the host's local reaction has already been weakened by hydrocortisone while its concentration may subsequently not be high enough to inhibit tumor growth completely.

DISCUSSION

The Walker tumor 256 originated from a mammary adenocarcinoma of the rat. The early history of this tumor in the animal and its morphology in tissue culture were exhaustively studied by Earle (*8*, *14*). The present findings in which a physiological rather than a morphological definition of fibroblasts has been employed are compatible with Earle's data. He described the following types of tumor cells: the typical round carcinoma cell; elongated and fibroblast-like cells, which "by closer examination are also carcinoma cells," and in addition smaller, slender spindle-shaped cells with an elongated slender nucleus and 1 to 2 nucleoli. As for the latter cells, "There was no evidence that they were of epithelial origin." Nevertheless, Earle regarded the cells of the stock tumor as well as those in tissue culture as carcinoma cells. In the present investigation, old tumors in tissue culture showed round, spindle-shaped, and ameboid forms of tumor cells, while young tumors, in addition to those tumor cells, showed fibroblasts of mesenchymal origin, which produced hyaluronic acid *in vitro*, a precursor of ground substance, and which derived from the host's subcutaneous tissue. The word "fibroblasts" has been used only to designate cells of mesenchymal origin producing interfibrillar substances, while the designation "spindle-shaped" has been applied to cells of mesenchymal origin as well as to epithelial and carcinoma cells.

Tumor tissues were often associated with production of enzymes capable of increasing the permeability of the ground substance of the host's tissue. Hyaluronidase appeared to be one means of increasing the permeability of the ground substance and of promoting the invasion of cancer cells. There was, however, no direct evidence that the hyaluronidase found in tumor cells actually hydrolyzed hyaluronic acid of the ground substance of the connective tissue surrounding the tumor. In the present work, the production of hyaluronic acid *in vitro* by fibroblasts growing from the host's subcutaneous tissue in response to tumor grafting and the hydrolysis of hyaluronic acid by the tumor extract, when the substrate was hyaluronic acid produced *in vitro* by those fibroblasts, strongly suggest that this substrate-enzyme pair plays a part in the host's reaction and in the invasiveness of this malignant tumor. Finally, the present findings are consistent with the fact that this tumor originates from an adeno-

carcinoma, since sarcomas have the potency of producing hyaluronic acid themselves, and it can hardly be assumed that the same cell would produce both hyaluronic acid and hyaluronidase. Therefore, sarcomas, *e.g.*, the Rous, Fujinami, and Jensen *sarcomas* (*5*), do not produce hyaluronidase, while carcinomas do. But there are malignant tumors, especially sarcomas, with a high degree of invasiveness which do not produce hyaluronidase. Analogically, there are also many kinds of spreading factors that are "hyaluronate-inactive" (*15*).

Hydrolysis of hyaluronic acid of connective-tissue ground substance by hyaluronidase of the tumor appears thus to be one way of invading neighboring tissue. But it can hardly be assumed that the production of hyaluronidase by tumor cells, especially by a typical anaplastic tumor like the Walker would constitute a new quality acquired by the tumor cells. This would imply the emergence of a new type of physiological differentiation and would be incompatible with generally assumed characteristics of cancer cells, such as partial loss of physiological differentiation with a tendency to progressive elimination of enzymes [7] rather than production of new ones absent in their stem cells. Although spreading factors are not always identical with hyaluronidase (*16*), the early findings that epithelial cells, but not cells of mesenchymal origin, enhanced spreading (*1*, *2*) suggest that the quality of hyaluronidase production by tumor cells is not acquired by them, but pre-exists in the normal stem cells of carcinomas. The present experiments with normal rat tissue demonstrate that extracts prepared from lung, intestine, liver, and kidney show hyaluronidase activity, while extracts from subcutaneous tissue and muscle (pectoralis) do not hydrolyze native hyaluronic acid. The spleen, however, does show hyaluronidase activity. This exception is now the subject of further investigation.

The production of hyaluronidase by carcinoma cells may thus be conceived not as differentiation of carcinoma cells by production of a new enzyme, but as the uncontrolled utilization of the quality of enzyme production inherent in the epithelial cell, the stem cell of carcinoma.

The function of hyaluronidase of normal epithelial cells and the possibility of its being instrumental in the local control of hyaluronic acid production by cells of mesodermal derivation can at present only be a conjecture. But the possibility of enzyme induction by the substrate hyaluronic acid in mammalian cells is now being investigated, since production of hyaluronic acid has been found to precede production of the enzyme.

REFERENCES

(*1*) Boyland, E., and McClean, D.: A factor in malignant tissues which increases the permeability of the dermis. J. Path. & Bact. 41: 553–565, 1935.

(*2*) Duran-Reynals, F., and Stewart, F. W.: The action of tumor extracts on the spread of experimental vaccinia of the rabbit. Am. J. Cancer 15: 2790–2797, 1931.

[7] In one case, however, increase of enzyme activity but not production of new enzymes, was reported (B.B. Westfall, *et al.*: J. Biophys. & Biochem. Cytol. 4: 567, 1958).

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(3) BALAZS, E. A., and VON EULER, J.: The hyaluronidase content of necrotic tumor and testis tissue. Cancer Res. 12: 326–329, 1952.
(4) KABAT, E. A.: Polysaccharide in tumors due to a virus of leucosis and sarcoma of fowls. J. Biol. Chem. 130: 143–147, 1939.
(5) PIRIE, A.: A hyaluronidase and a polysaccharide from tumours. Brit. J. Exper. Path. 23: 277–284, 1942.
(6) GROSSFELD, H., MEYER, K., GODMAN, G., and LINKER, A.: Production of mucopolysaccharides in tissue culture. J. Biophys. & Biochem. Cytol. 3: 391, 1957.
(7) GROSSFELD, H.: Studies on production of hyaluronic acid in tissue culture. The presence of hyaluronidase in embryo extract. Exper. Cell Res. 14: 213, 1957.
(8) EARLE, W. R.: A study of Walker rat mammary carcinoma 256 *in vivo* and *in vitro*. Am. J. Cancer 24: 566–612, 1935.
(9) MCCOY, T. A., and NEUMAN, R. E.: The cultivation of Walker carcinosarcoma 256 *in vitro* from cell suspensions. J. Nat. Cancer Inst. 16: 1221–1229, 1956.
(10) MCCLEAN, D.: Studies on diffusing factors. Methods of assay of hyaluronidase and their correlation with skin diffusing activity. Biochem. J. 37: 169, 1937.
(11) GROSSFELD, H.: Metachromasia in the living cell. Proc. Soc. Exper. Biol. & Med. 86: 81–83, 1954.
(12) SIMPSON, W. L.: Mucolytic enzymes. Ann. New York Acad. Sc. 52: 1124, 1950.
(13) GROSSFELD, H., and RAGAN, C.: Action of hydrocortisone on cells in tissue culture. Proc. Soc. Exper. Biol. & Med. 86: 63–68, 1954.
(14) EARLE, W. R.: Walker 256 rat mammary carcinoma *in vitro*. Further observations. Arch. Path. 27: 80–87, 1939.
(15) HECHTER, O.: Mechanism of spreading factor action. Ann. New York Acad. Sc. 52: 1028, 1950.
(16) MEYER, K., CHAFFEE, E., HOBBY, G. L., and DAWSON, M. H.: Hyaluronidases of bacterial and animal origin. J. Exper. Med. 73: 309–326, 1941.

PLATE 63

FIGURE 1.—Living culture of Walker tumor 256 explanted on 13th day after subcutaneous transplantation. Dense growth of tumor cells only.

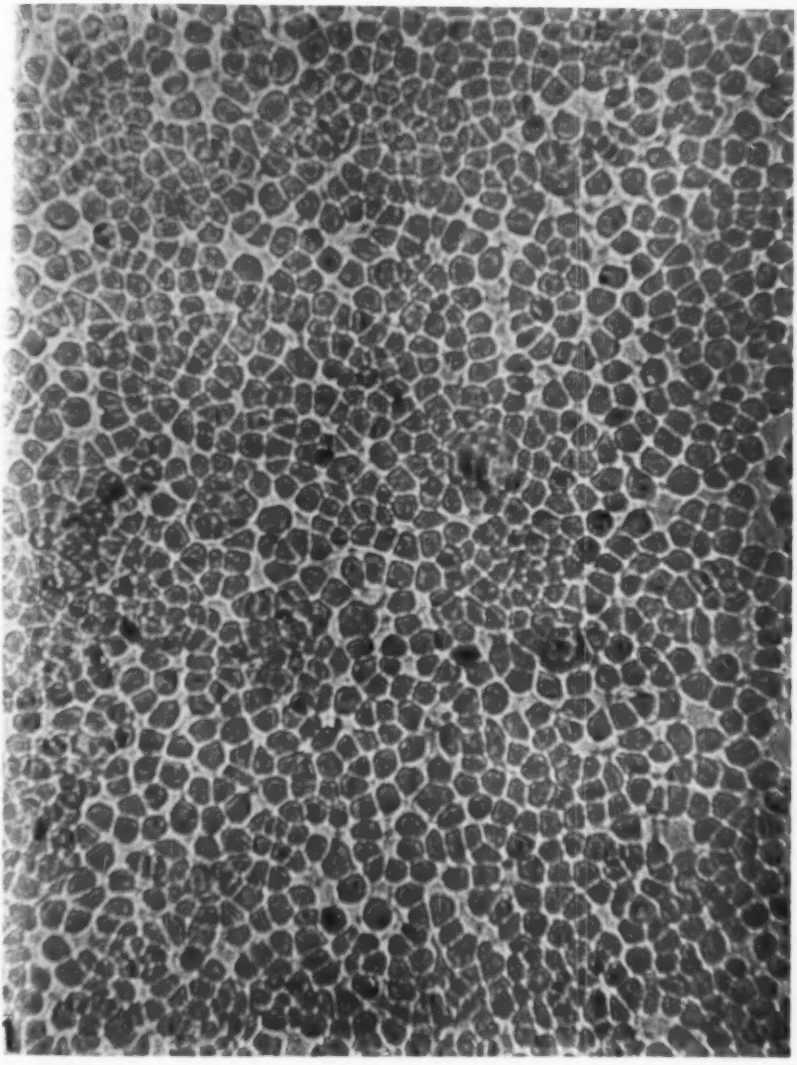

PLATE 64

FIGURE 2.—Living culture of Walker tumor 256 explanted on 10th day after subcutaneous transplantation. Growth of tumor cells only.

FIGURE 3.—Living culture of Walker tumor 256 explanted on 12th day after subcutaneous transplantation. Growth of tumor cells only.

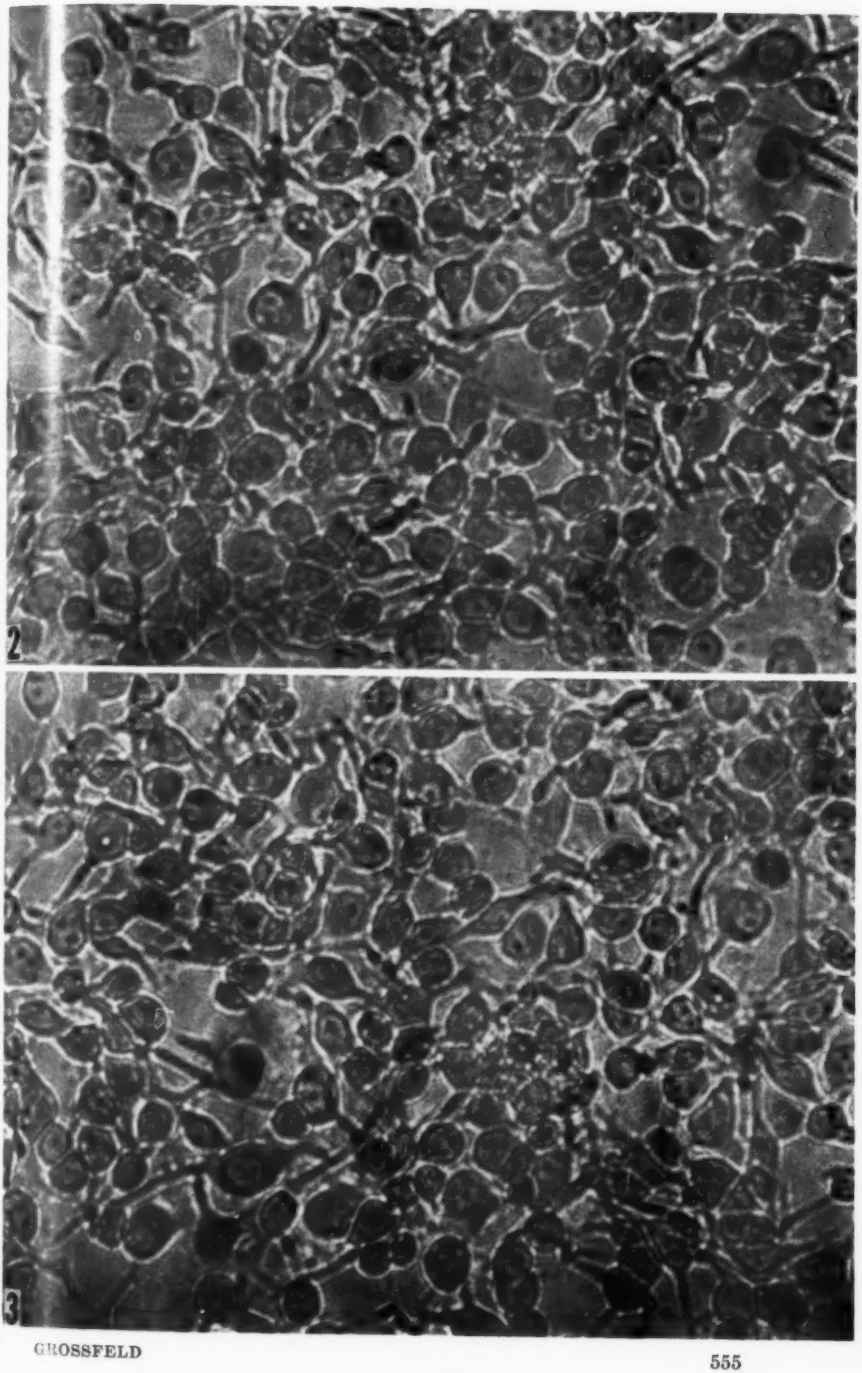
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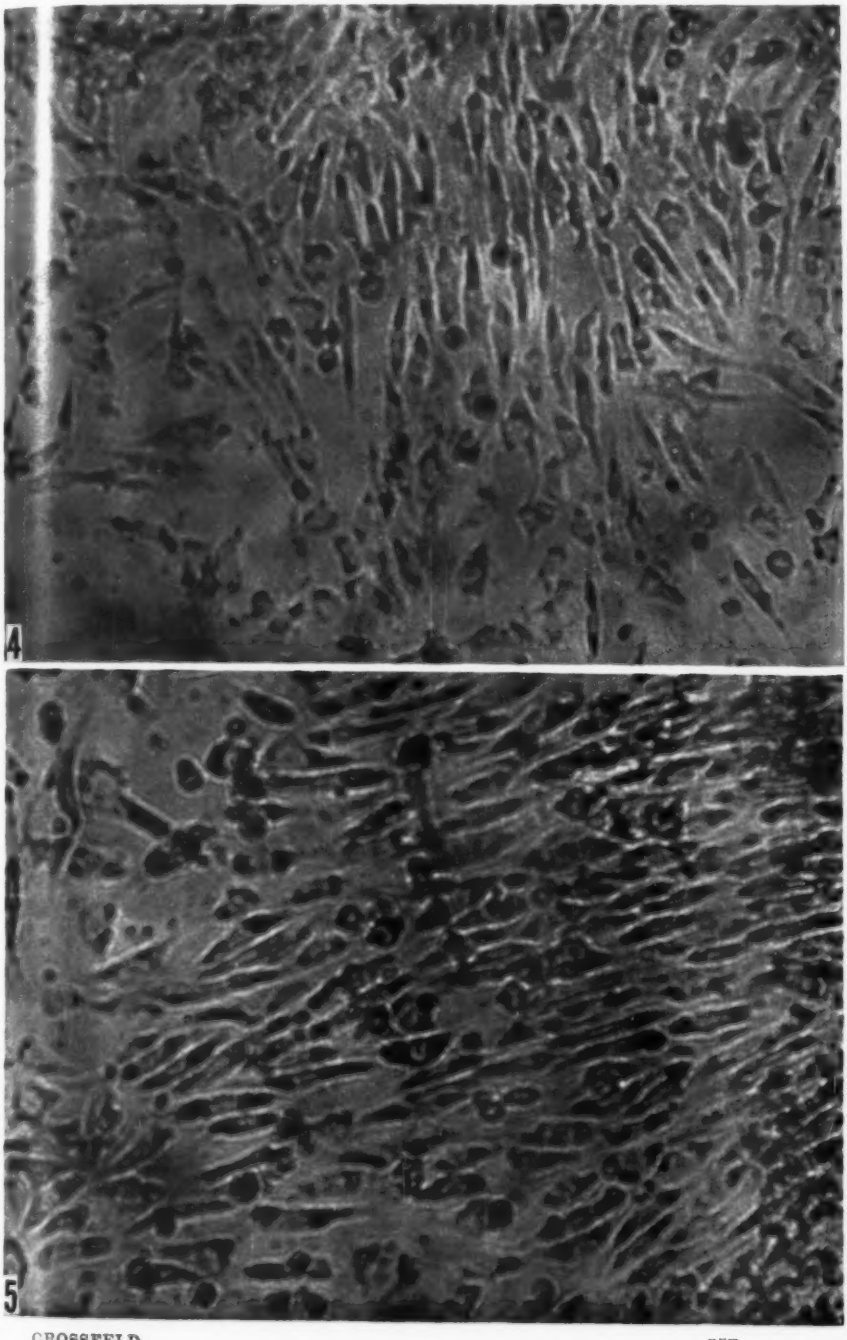
PLATE 65

FIGURE 4.—Living culture of Walker tumor 256 explanted on 5th day after subcutaneous transplantation. Growth of fibroblasts producing hyaluronic acid *in vitro*.

FIGURE 5.—Living culture of Walker tumor 256 explanted on 6th day after subcutaneous transplantation. Growth of fibroblasts producing hyaluronic acid *in vitro*.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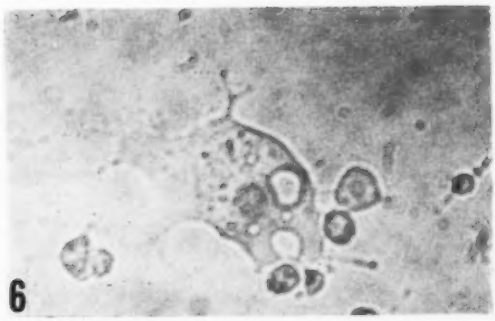

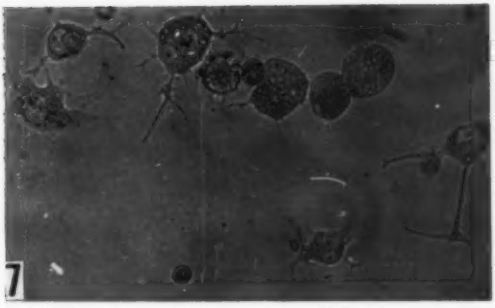

FIGURES 6 and 7.—Living cells from Walker tumor 256 showing formation and branching of pseudopodia.

Influence of Adrenocortical, Androgenic, and Anabolic Hormones on the Development of Carcinoma and Cirrhosis of the Liver in A × C Rats Fed *N*-2-Fluorenyldiacetamide [1, 2]

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SUMMARY

An experimental model was developed to detect the role of various endocrine organs and their hormones in the induction of cirrhosis and carcinoma of the liver. This model uses inbred A × C rats, fed 0.025 percent *N*-2-fluorenyldiacetamide in a semi-synthetic diet according to a regimen that produces less than 100 percent of tumors in susceptible intact males surviving the induction period of 25 weeks. With this model it was found that: 1) Male rats were far more susceptible to cirrhosis and carcinoma of the liver than female rats. 2) Castration protected male rats against cirrhosis but showed little effect on tumor induction. 3) Simultaneous administration of testosterone restored the susceptibility to cirrhosis in castrated males and resulted in both cirrhosis and carcinoma in intact and castrated female rats. 4) Castrated female rats, given testosterone 4 months after the carcinogen was stopped, failed to develop either cirrhosis or carcinoma. 5) Adrenalectomy seemed to offer some protection against cirrhosis but not against tumors. Adrenalectomized female animals given testosterone were far more susceptible to tumors than intact females given testosterone. 6) Deoxycorticosterone acetate had a striking protective effect both against cirrhosis and carcinoma in male rats. 7) Norethandrolone, an anabolic hormone with minimal androgenic activity in the dosage administered, was equally potent to testosterone in promoting cirrhosis and carcinoma of the liver in all the groups tested. 8) The presence of anabolic hormone, either testosterone or norethandrolone, affected not only the incidence but also the morphology of the tumors, since poorly differentiated tumors were found only in animals subjected to exogenously administered testosterone or norethandrolone or endogenous testosterone. These findings suggest a close correlation between carcinogenesis and the level of protein metabolism of the liver cells at the time the carcinogen is administered. The possible relationship of protein metabolism to a mechanism of carcinogenesis is described.—J. Nat. Cancer Inst. 27: 559–595, 1961.

IT IS well known that hepatocellular carcinoma occurs more frequently in men than in women and that it is generally associated with cirrhosis

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[3] This research was started at the University of Kansas and was completed at the University of Maryland, the *present address* of the authors.

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(*1*, *2*). A similar association with cirrhosis and preferential sex incidence has also been reported by a number of investigators in rats fed various carcinogens (*3–8*). Castration of rats fed *N*-2-fluorenylacetamide has proved to be some protection against cirrhosis and carcinoma of the liver, but the incidence of both lesions could be restored by administration of testosterone propionate during the period the carcinogen was fed (*9*). Thyroidectomy prevented the development of neoplasms of the liver (*10*) in rats exposed to *N*-2-fluorenylacetamide. As might be expected, hypophysectomy protected rats from hepatic carcinogenesis (*11–15*) with 3′-methyl-4-dimethylaminoazobenzene or *N*-2-fluorenyldiacetamide, but there are conflicting reports concerning the effect of adrenalectomy (*13*, *16–18*) when azo dyes are used as carcinogens.

It seemed that some of the reported differences could be due to differences in the procedures used; in particular, the overwhelming dosage of a powerful carcinogen might well mask the role of hormones. Indeed, a number of experiments with 3′-methyl-4-dimethylaminoazobenzene or *N*-2-fluorenyldiacetamide have failed to reveal even a difference in susceptibility between the sexes (*13*, *19*). Consequently the first efforts were to develop a model in which most of the intact male animals lived long enough to develop large tumors, at times with metastases. The dosage was regulated so that somewhat less than 100 percent of the animals developed tumors.

MATERIALS AND METHODS

Inbred A × C rats weighing from 150 to 200 g were used; a few animals weighed slightly over 200 g. They were fed a semisynthetic diet [5] designed by Morris to which *N*-2-fluorenyldiacetamide (2-FdiAA) was added to make 0.025 percent. The dosage of carcinogen administered to each group of animals was regulated by varying the time on the carcinogen. Previous experiments had revealed the desirability of alternating periods of carcinogen administration with short "rest periods" on the semisynthetic diet without carcinogen (*9*). Throughout the experiment water was available *ad libitum*.

To define the desired dosage more precisely, the length of time the carcinogen was administered was varied among 4 groups of animals. In each group the rats were fed the carcinogen-containing diet for 4 weeks followed by 1 week of the same diet without carcinogen. At that time the animals were returned to the carcinogen-containing diet for another 4-week period followed by 1 week of diet without carcinogen. Thus the diet was alternated until 8, 10, 12, or 16 weeks of carcinogen feeding had been completed according to the preselected time for the respective groups. After completion of the last period on the carcinogen, the animals were

[5] Morris diet No. 272 consisted of 300 g commercial casein, 2275 g skim-milk powder, 6152 g ground, hard spring wheat (12–13.5% protein), 200 g brewer's yeast, 200 g desiccated liver, N.P. (Wilson Laboratories, Chicago, Ill.) 140 g sodium chloride, 10 g ascorbic acid, 13 g ferric citrate, 100 g cod-liver oil, and 610 g corn oil.

maintained on the basic diet without carcinogen until their death or until the experiment was terminated at the end of 65 weeks.

All animals were weighed weekly, and an exploratory laparotomy with biopsy of the liver was performed on each animal at 6- to 8-week intervals to trace the development of lesions and determine the earliest appearance of tumors. This provided material for transplantation, which will serve as the basis of a separate report. Also, at the time of death of the animals, autopsies provided additional material for microscopic examination and transplantation.

RESULTS

Previous studies of carcinogenesis of the liver (*20–26*) as well as those derived from the present experiments all revealed a similar progression of morphologic changes from normal through various hyperplastic parenchymal proliferations to neoplasia. Simultaneously there was often proliferation of small bile ducts and minimal accompanying fibroblastic activity progressing to apparent isolation of lobules and in more florid instances to isolation of liver cells (figs. 1 and 2). Some larger bile ducts at times showed similar proliferative activity in localized areas, often with the formation of cystic lesions made up of a group of cystic ducts. Ceroid pigment was usually also evident in macrophages in the portal areas.

Hyperplasia of parenchymal cells seemed the obvious forerunner of hepatocellular carcinoma; hence, for purposes of evaluation of the findings in the livers of each of the experimental animals, they were classified and tabulated as showing:

1) *No hyperplasia:* This included also animals that showed basophilia of the cytoplasm of the periportal cells or the earliest hyperplastic change diffusely in the periportal zones.
2) *Hyperplasia:* These were animals with livers containing multiple small foci or larger areas of hyperplasia which were distinct from the surrounding liver and which often showed somewhat different architectural arrangement from the normal lobule (figs. 3, 4, 5, and 6).
3) *Hyperplastic nodules:* These were animals in which the hyperplasia had progressed to the formation of clearly defined nodules of parenchymal cells with distinct compression of surrounding parenchyma (figs. 7 and 8). The architecture in these nodules varied from well-organized adult cords to a disorderly pattern of growth, at times with atypicality of cells but of such a degree that it was not considered malignant (fig. 9).
4) *Small hepatoma:* These hepatomas represented the earliest and smallest lesions that could be morphologically accepted as malignant; yet, by definition, they were limited to lesions no more than 5 mm in greatest diameter (fig. 10).
5) *Well-developed hepatocellular carcinoma or occasionally cholangiohepatoma:* These animals had malignant tumors of parenchymal cells which were well differentiated, poorly differentiated, or anaplastic growths (figs. 12 and 15). Occasionally there were cholangiomatous areas in the hepatocellular carcinomas (fig. 14). Invasion of portal or hepatic veins was frequent and in some animals multiple emboli or metastases were seen in the lungs (figs. 11 and 13). In some animals, multiple implants were also found over the peritoneal surfaces.

For the purposes of tabulation or graphing (table 1), only the most advanced lesion of each liver was used, though in most instances "lesser lesions" accompanied the more advanced hyperplastic or neoplastic lesions. The results of this preliminary study, shown in table 1, reveal that 8 weeks of the carcinogen were inadequate to produce either cirrhosis or tumors. Ten weeks of carcinogen feeding produced cirrhosis and 2 small hepatomas, but 16 weeks on the carcinogen were required for more than 50 percent of intact male rats to develop hepatocellular carcinoma. The earliest small hepatoma in this group appeared 25 weeks after the start of the carcinogen-containing diet. Sixteen weeks of carcinogen feeding were adopted for the model. Unfortunately the procedure of periodic laparotomy and biopsy did not reveal many of the tumors, particularly in their early stages. Hence, data collected in this manner could not be used to determine the latent periods for each of the experimental groups.

TABLE 1.—Preliminary study with intact male rats to determine the desirable duration of administration of the carcinogen-containing diet*

Time on carcinogen (weeks)	Number of animals in group	Number of animals with lesions shown							
		No hyperplasia	Hyperplasia	Nodules	Small hepatoma	Hepatocellular carcinoma	Metastases	Total animals with liver tumors	Cirrhosis
8	14	8	6	0	0	0	0	0	0
10	10	0	3	5	2	0	0	2	7
12	10	0	1	4	0	5	1	5	8
16	11	0	1	3	1	6	1	7	9

*Animals are classified according to the most advanced lesion found. The presence of metastases and/or cirrhosis is also indicated. The average latent period in the 12- and 16-week groups was 51 weeks without significant difference between the two groups.

EVALUATION OF THE ROLE OF CERTAIN ENDOCRINE ORGANS AND HORMONES IN CIRRHOSIS AND HEPATIC CARCINOGENESIS

After selection of the basic procedure, the influence of various endocrine glands or exogenously administered hormones was tested. Endocrine organs were removed 2 weeks before the carcinogenic diet was started. In groups of animals given hormone it was administered simultaneously with the start of the carcinogenic diet, with one notable exception in which a group of castrated female rats was given the carcinogen in the diet and testosterone was not administered until 4 months after the carcinogen was withdrawn.

In 6 or 8 weeks after the start of the experiment, an exploratory laparotomy was performed on each group, with biopsy of the liver of the most advanced lesion visible on the surface of the liver. Biopsies generally consisted of wedge-shaped bits of liver measuring about 3 × 3 × 5 mm

on each edge. This was repeated several times in each animal to determine the time of the earliest appearance of tumor and provide material for microscopic examination and transplantation studies. All animals at the time of death or as soon as possible thereafter were autopsied, and tissue was obtained for transplantation and representative microscopic sections. The gross findings at autopsy are summarized in table 2. In those animals in which endocrine organs had been removed, a thorough search, both grossly and microscopically, was made for residual, regenerated, or ectopic endocrine tissue.

The multiplicity of findings in the different experimental groups made it necessary to assign numerical values to each of the items considered in the evaluation for determination of significant differences between the groups. Items included were: type of hyperplastic or neoplastic microscopic lesion, the number and size of tumors in the liver, the over-all weight of the liver, and the number of metastases in the lungs with a correction for length of survival. (*See* Appendix for details of scoring procedure.) With this system, the numerical value for each animal was calculated and comparison of the statistical significance of the differences between the means of the two groups being compared was calculated. When differences between two groups are referred to subsequently in the text, they had P values of 0.01 or less unless otherwise stated. Table 3 gives the mean scores for each group with the ranges and standard errors.

Gonads and Sex Hormones

The first experiment was an attempt to re-evaluate the role of sex and sex hormones in cirrhosis and carcinogenesis. In this experiment nine groups of animals were studied. They consisted of intact males and females, intact males and females given testosterone propionate, castrated males and females, castrated males and females given testosterone propionate, and finally a group of castrated females in which the administration of testosterone propionate was delayed until 4 months after the carcinogen was stopped. In those animals receiving testosterone, a single pellet containing 1 part of testosterone propionate and 3 parts of cholesterol was implanted subcutaneously in the interscapular area. The dose consisted of 20 mg of testosterone propionate per 100 g of rat or 80 mg of pellet per 100 g. All surviving animals were killed at the end of 40 weeks.

The intact males and females both lost considerable weight during the initial 4 weeks on the carcinogen, but rapidly regained the lost weight when the carcinogen was removed from the diet during the 5th week. Thereafter the animals continued to gain or maintain their weight despite reinstatement of the carcinogen-containing diet. In groups of intact animals the administration of testosterone had little effect on the weight curves except for a slight delay in the initial weight loss in the females.

The castrated males and females initially lost weight in a manner similar to the intact animals and generally followed the same course

TABLE 2.—Summary of gross lesions of the liver and lungs*

Group	Number of animals	Average liver weight (g)	Average number of tumors per liver	Usual tumor diameter (cm)	Lung metastases	Average survival (weeks)
Castrated males given testosterone	10	35.5	More than 3	Up to 7.0	Rare†	39
Adrenalectomized males	15	29.5	More than 3	Up to 7.0	Massive	52
Adrenalectomized females given testosterone	12	33.2	More than 3	Up to 4.5	Many	57
Castrated males given norethandrolone	8	27.5	3	1.5 × 2.5	None†	37
Adrenalectomized males given 2 mg cortisone	10	26.6	2–3	3.0	Few†	38
Adrenalectomized, castrated females given testosterone	14	26.2	2–3	1.5 × 4.5	Rare	54
Intact males (2 groups)	11	20.0	2	2.0 × 4.5	Rare	56
	13	15.5	2	1.0 × 1.5	None	39
Intact males given testosterone	10	24.1	1	1.5 × 2.0	None	42
Intact males given norethandrolone	14	17.6	1	1.5 × 2.0	None	32
Intact females given norethandrolone	13	18.8	1	1.5 × 2.0	None	41
Adrenalectomized males given 1 mg cortisone	10	16.5	1	1.5 × 1.8	None	37
Castrated males	12	15.1	1	1.5 × 2.0	None	60
Castrated females given norethandrolone	10	16.3	1	1.5 × 2.0	None	40
Castrated females given testosterone	8	16.5	1	1.0	None	42
Intact females given testosterone	13	11.6	1	1.0	None	40
Adrenalectomized males given deoxycorticosterone acetate	7	11.4	None	—	—	40
Adrenalectomized, castrated females	12	10.4	None	—	—	63
Castrated females	14	9.3	None	—	—	58
Adrenalectomized females	14	8.0	None	—	—	60
Intact females	14	8.7	None	—	—	62

*Liver weight of control adult animals on laboratory pellets is 10.9 g for males and 7.2 for females. The groups of animals are arranged in an approximate order with the most susceptible at the top and the least or nonsusceptible at the bottom. The order is similar but slightly altered from that obtained with the scoring procedure, as seen in table 4.

†The animals in these groups died earlier in the experiment, which may have influenced the results.

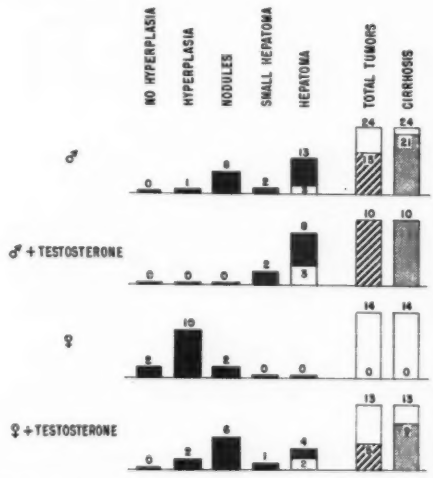
as intact animals, but the administration of testosterone to these two groups delayed the initial weight loss until the 2d month on the carcinogen. After this loss the animals returned to a more normal weight and continued to gain or maintain their weight.

Text-figures 1 and 2 and tables 2 and 3 confirm the difference in susceptibility of males and females to cirrhosis and hepatocellular carcinoma (figs. 16 and 19). Castration of the males produced a minimal reduction in the incidence of hepatocellular carcinoma, but the tumors tended to be fewer and smaller than in intact males (fig. 17) and there were no metastases. More striking was the complete absence of cirrhosis.

TABLE 3.—Mean score* for each group listed in table 2

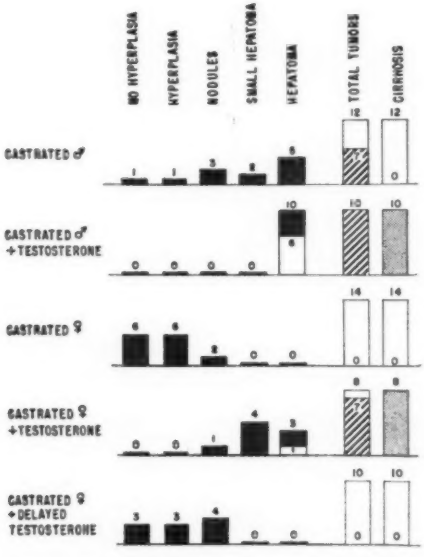
Group	Number of animals	Scores		Standard error
		Range	Mean	
Castrated males given testosterone	10	58–513	277. 7	±45. 1
Adrenalectomized males	13	13–546	197. 9	±48. 2
Adrenalectomized males given 2 mg cortisone	10	22–580	185. 7	±43. 0
Adrenalectomized females given testosterone	12	17–407	161. 5	±38. 3
Castrated males given norethandrolone	8	47–313	145. 0	±32. 1
Intact males given testosterone	10	34–423	125. 0	±38. 5
Castrated females given norethandrolone	10	13–315	111	±37. 6
Adrenalectomized, castrated females given testosterone	14	4–389	105. 0	±30. 3
Intact males given norethandrolone	14	24–330	89. 7	±21. 5
Intact males	24	12–300	76	±15. 3
Intact females given norethandrolone	11	33–182	65	±13. 5
Castrated females given testosterone	9	31–200	54. 7	±18. 7
Adrenalectomized males given 1 mg cortisone	10	8–216	53. 2	±6. 2
Castrated males	12	4–86	39. 4	±8. 1
Intact females given testosterone	13	10–71	30	±5. 9
Adrenalectomized males given deoxycorticosterone acetate	7	4–70	17	±9. 0
Adrenalectomized, castrated females	13	4–30	13	±2. 2
Intact females	14	6–15	10	±0. 68
Castrated females	13	4–15	9. 2	±1. 0
Adrenalectomized females	14	4–17	9	±1. 3

*See Appendix for scoring procedure.



TEXT-FIGURE 1.—Intact animals given testosterone. In all text-figures only the most advanced lesion in a given animal is graphed. Cirrhosis is included adjacent to the total tumors to make a comparison between the effects of hormones on both lesions. The number above each *black bar* is the total number of animals developing such lesions. Under hepatomas the *white area* designates the number of animals with metastases. Total tumors and cirrhosis show total number of animals in the group as well as the number with those lesions.

It is interesting that in approximately 50 percent of the castrated male animals there was cirrhosis early in the course, as shown by liver biopsy, but this had completely disappeared by the time of autopsy. The susceptibility to cirrhosis was restored by implantation of a pellet of testosterone propionate subcutaneously at the beginning of the experiment, and tumors in these animals were larger, often confluent, and metastasized. This dosage of testosterone is probably greater than physiologic, and the incidence of hepatocellular carcinoma was 100 percent, with 60 percent of the animals showing metastatic lesions. This increased level of testosterone seemed to increase the incidence of cirrhosis and hepatocellular carcinoma in intact males, but statistically the difference was not significant. Curiously, in intact males given testosterone the tumors were not nearly so large, or numerous, nor were the metastases impressive as in the castrated males given testosterone. Although the P value between these two latter groups was only 0.021, the results suggest a slight protective effect of the testis.



TEXT-FIGURE 2.—Castrated animals given testosterone.

As might be expected, castration of the females showed little change from the intact females; in both, the changes in the liver were minimal. Interestingly, testosterone pellets in intact females produced cirrhosis and hepatocellular carcinomas (fig. 18) and increased the number of hyperplastic nodules but did not quite produce the same picture that was seen in intact males, which suggested a possible inhibitory effect of the ovaries (P value 0.012). Upon removal of the ovaries and administration of testosterone, the incidence of cirrhosis and tumors exceeded

that of intact males and rivaled that of castrated males given testosterone, but there was little comparison between those two groups when one considered the size and number of hepatic tumors and the abundance and size of the metastases in the animals. In these respects the findings were more like those of castrated males without testosterone. The castrated males given testosterone were far more impressive. These differences re-emphasize a distinct difference between males and females, even after castration.

The castrated males receiving testosterone died somewhat earlier than the remaining animals and several died as a result of peritoneal hemorrhage from a tumor. The latter was not observed in the remaining groups. Microscopically, the tumors of the liver were usually well-differentiated hepatocellular carcinomas. In the intact males there was 1 undifferentiated hepatocellular carcinoma and 2 cholangiohepatomas; in intact males receiving testosterone, 2 poorly differentiated hepatocellular carcinomas; in castrated males given testosterone, 3 poorly differentiated hepatocellular carcinomas, and 1 cholangiohepatoma; and in one castrated female given testosterone an undifferentiated hepatocellular carcinoma. Thus testosterone also appeared to have some effect on the cellular pattern as it tended to produce more undifferentiated tumors.

In one group of castrated females the administration of testosterone was delayed until 4 months after the diet containing the carcinogen was stopped. In this group neither cirrhosis nor tumors developed, which indicated the necessity of administering testosterone propionate during the period of carcinogen administration. The significance of this finding will be discussed subsequently.

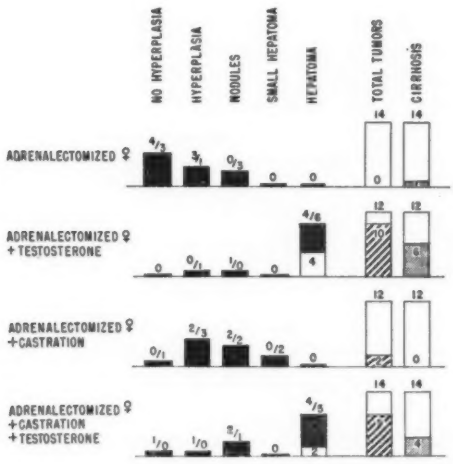
Adrenal Glands Versus Gonads and Testosterone Propionate

Five groups of animals were studied including adrenalectomized males and females, adrenalectomized and castrated females, adrenalectomized and castrated females given testosterone propionate, and adrenalectomized females given testosterone without castration. Surviving animals in each group were killed at the end of 65 weeks. A sixth group of adrenalectomized castrated males died off too rapidly and had to be dropped.

Adrenalectomy and/or the 1 percent salt in the drinking water for either sex seemed to protect somewhat against weight loss compared to intact animals. Even these groups tended to lose some weight initially when given carcinogen-containing diet and to regain their weight as they became adapted or were returned to the diet without carcinogen. The administration of the testosterone to adrenalectomized or adrenalectomized and castrated females not only abolished the initial weight loss but actually produced a rather notable early weight gain.

The findings in these animals are shown in text-figures 3 and 4 and tables 2 and 3. In this experiment the group of adrenalectomized females given

testosterone died somewhat earlier with extensive tumor involvement so that before 62 weeks all the animals were dead with massive tumors and extensive pulmonary metastases. The adrenalectomized females given testosterone and the adrenalectomized castrated females given testosterone had more tumors, larger tumors, and more metastases than did the castrated females receiving the same hormone. The incidence of cirrhosis, however, in these same groups was directly opposite that of the tumors. Microscopically the tumors were all well-differentiated hepatocellular carcinomas.



TEXT-FIGURE 3.—Adrenal glands versus gonads and/or testosterone. In this and the next text-figure *upper number* above each bar represents the animals without adrenal regeneration or ectopic adrenocortical tissue and *lower number* those with adrenocortical tissue.

Adrenocortical regeneration or ectopic adrenocortical tissue was usually tiny and often only microscopic. Eight of 14 adrenalectomized males not receiving hormones showed such adrenocortical tissue. In the females it was seen in 7 of 14 adrenalectomized and 8 of 12 adrenalectomized and castrated animals. The administration of testosterone did not alter the frequency of regeneration in that 7 of 12 adrenalectomized rats and 7 of 14 adrenalectomized castrated females bore such tissue. By contrast, the administration of adrenocorticoids after adrenalectomy was very effective in preventing adrenocortical regeneration.

Adrenalectomized Animals Given Corticoids

For comparison with the effects of adrenalectomy alone, three groups of adrenalectomized male rats were studied. The first group was given 1 mg of a saline suspension of cortisone acetate,[6] and the second, 2 mg of

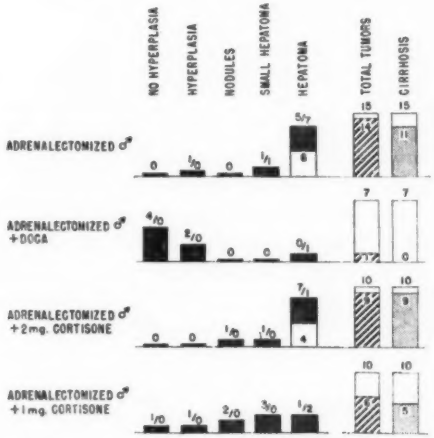
[6]The Upjohn Co., Kalamazoo, Mich., as Cortone Acetate.

cortisone acetate subcutaneously every 3 days for the duration of the experiment. The third group had 75 mg pellets of pure crystalline deoxycorticosterone acetate (DOCA)[7] implanted subcutaneously in the interscapular region at the beginning of the experiment and a second such pellet 6 months later. These animals were maintained continuously on 1 percent sodium chloride in their drinking water just as the groups of adrenalectomized animals without corticoid supplementation. The experiment was terminated at 40 weeks.

Again the adrenalectomy and/or intake of 1 percent salt water prevented the initial weight loss except in the group given 2 mg of cortisone. In this latter group the weight curve was much like that of intact male animals. By the 12th week the starting weight was regained and from then on there was a progressive weight gain similar to the other groups in this experiment.

The findings are shown in text-figure 4 and tables 2 and 3. Adrenalectomized males had extensive liver involvement by tumor (fig. 20) and metastases to the lungs were usually massive (fig. 21). Livers of animals given DOCA were normal (fig. 22). The incidence of tumors in animals receiving 2 mg of cortisone was similar to that in the adrenalectomized animals without hormones, but the gross characteristics more closely resembled those of intact males without hormones.

There was 1 undifferentiated hepatocellular carcinoma in an adrenalectomized male and 1 in an adrenalectomized male given the 2 mg dose of cortisone. This total was somewhat smaller than the number of such undifferentiated tumors in the animals receiving testosterone. There were no cholangiohepatomas. Adrenal regeneration or ectopic adrenocortical tissue was noted in 2 of 10 rats receiving the 1 mg cortisone dose, 1 of 10 receiving the 2 mg cortisone dose, and 1 of 7 given DOCA.



TEXT-FIGURE 4.—Adrenalectomized animals receiving corticoids.

[7] Schering Corp., Bloomfield, N.J., as "Cortate" pellets.

Norethandrolone Versus Testosterone

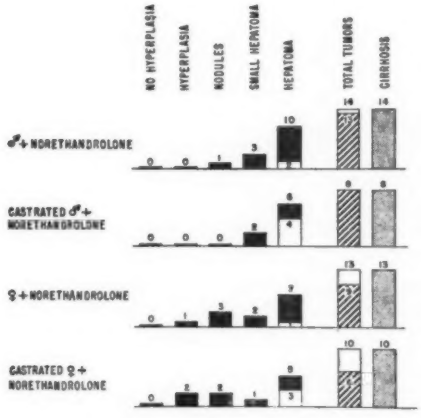
It also seemed desirable to determine the effects of adrenocortical anabolic hormone. The readily available compound that might be regarded as comparable is norethandrolone.[8] It has predominant anabolic activity with minimal androgenic activity (*27*). The norethandrolone was dispersed in physiologic saline to make a 10 percent suspension. For comparison with testosterone propionate, identical groups of animals were prepared and given 0.1 cc of the suspension containing 1 mg of norethandrolone, subcutaneously every 3 days, for the duration of the experiment. Unfortunately, a group of adrenalectomized males given norethandrolone in this experiment died very early and had to be abandoned.

Intact males given norethandrolone or testosterone had almost identical weight curves with an initial loss followed by progressive rise. Castrated males given either testosterone or norethandrolone showed a delay in weight loss until after the 12th week of the experiment, and norethandrolone produced a somewhat more noticeable initial loss with earlier death of the animals than testosterone.

In females, either intact or castrated, given testosterone propionate there was considerable delay in weight gain, but this period was much shorter in the comparable animals given norethandrolone.

The findings in these groups are given in text-figure 5 and tables 2 and 3, which by comparison with text-figures 1 and 2 reveal surprising correspondence between the effects of norethandrolone and those of testosterone. A representative liver is illustrated in figure 23.

In the intact male group given norethandrolone there was 1 cholangiohepatoma and 1 undifferentiated hepatocellular carcinoma. The remainder of the hepatic tumors in this group were well differentiated.



TEXT-FIGURE 5.—Animals receiving anabolic hormone.

[8] Kindly supplied by Searle and Co., Chicago, Ill., as "Nilevar"®.

Primary Tumors Other Than Liver

Other miscellaneous tumors are listed in table 4. Details of the carcinosarcomas of the salivary glands and the endometrial sarcomas of the uterus have been previously reported (*28*).

TABLE 4.—Sites of primary tumors other than liver. Almost all these tumors were found at the termination of the experiment and did not preclude the finding of hepatomas

Group	Tumors	Total number of animals
Castrated females given norethandrolone	3 sarcomas, endometrium 2 carcinosarcomas, salivary gland	10
Castrated males given testosterone	1 carcinosarcoma, salivary gland	10
Adrenalectomized females	1 poorly differentiated sarcoma, subcutaneous tissue, neck	14
Adrenalectomized females given testosterone	2 spindle-cell sarcomas, thoracic wall* 1 ossifying fibroma, groin 1 fibrosarcoma, area of pellet 1 adenocarcinoma, small intestine	12
Adrenalectomized and castrated females	1 spindle-cell sarcoma, thoracic wall 1 rhabdomyosarcoma, groin	12
Adrenalectomized and castrated females given testosterone	1 osteogenic sarcoma, thoracic wall* 1 poorly differentiated sarcoma, groin, with lung metastases 1 poorly differentiated sarcoma, thoracic wall	14
Castrated males	1 basophil adenoma, pituitary	12
Castrated females	1 nodule sparsely granulated basophils, pituitary	14

*Transplanted subcutaneously in A × C rats for 8 generations.

DISCUSSION

The studies of the role of gonads and/or testosterone confirm the procarcinogenic effect of the male sex hormone whether endogenous or exogenous. Comparison of the findings in intact versus castrated animals given testosterone propionate suggests a protective effect of the ovaries and, curiously also, possibly of the testes. Poorly differentiated or undifferentiated tumors were seen only in males or animals receiving anabolic hormone, which corresponded to the findings of Sidransky *et al.* (*29*). Studies of the effects of progesterone and estrogens in this model are being carried out (*30*). A striking finding was the complete protection of castration against cirrhosis in male animals with little change in the over-all incidence of neoplastic lesions, though the latent period was longer, the lesions were smaller, usually solitary, and better differentiated.

Studies of the effect of adrenalectomy revealed a distinctly greater susceptibility to hepatic carcinogenesis and metastases in adrenalectomized male rats and in adrenalectomized female rats given testosterone when compared to corresponding intact animals. In female animals, carcinoma began to appear when castration was performed in addition

to adrenalectomy. Yet adrenalectomy or castration alone was insufficient to allow the induction of tumors in female rats. Hence it would seem that the protective effects of the ovaries and adrenal glands in female rats were additive. More well-developed tumors were seen with exogenously administered testosterone propionate in adrenalectomized than in castrated females, so that the protective effect of the adrenal glands would seem somewhat greater than that of the ovaries, though the P value was only 0.023 between these two groups.

In another experiment both biopsied and nonbiopsied groups of animals were studied (*30*). The nonbiopsied rats developed a significantly higher percentage of both tumors ($P = 0.002$) and cirrhosis than did the biopsied animals. This is further evidence that the role of the adrenal gland in hepatic carcinogenesis is protective.

The protective effect of the adrenal glands was previously reported by Griffin *et al.* (*13*) with azo-dye carcinogenesis, but it is contrary to the findings of Symeonidis *et al.* (*16*) and of Eversole and Da Vanzo (*17, 18*). Each of the latter reported a decreased incidence of hepatic tumors in rats after adrenalectomy. Symeonidis further indicated that hepatic tumors could be found when the adrenal cortex regenerated from ectopic foci, as it did in approximately 50 percent of rats. In our experiments, also, there was regeneration of adrenocortical tissue in approximately 50 percent of rats in the retroperitoneal fat. But in these experiments there seemed to be no correlation of hepatic tumors with the regeneration of adrenocortical tissue, which suggested that even with regeneration there was a deficiency of the adrenocortical factor or factors concerned in the protection against carcinogensis.

In an attempt to distinguish a cortical factor which might be responsible for the protective effect, mineralocorticoid and glucocorticoid hormones were administered to adrenalectomized animals and the difference in results was striking. Only one of the rats receiving DOCA developed a carcinoma while most of the animals given cortisone, whether in 1 or 2 mg doses every 3 days, developed hepatocellular carcinoma. The protective effect of DOCA in adrenalectomized rats is the one finding that all investigators seem to agree upon, although Eversole suggests it is a factor of dosage. We would be inclined to agree, but not so much on the role of the dosage of deoxycorticosterone as on a dosage of carcinogen which does not swamp all other operative factors.

It is interesting that in addition to the protective effect of DOCA against tumors it also offered complete protection against cirrhosis. Yet it should be pointed out that in all adrenalectomized animals the incidence of cirrhosis of the liver was equal to or less than the incidence of tumors, in contrast to practically all other groups in which cirrhosis almost always equaled or excelled the incidence of tumors, with the outstanding exception of the group of castrated males mentioned.

As an aside it should also be mentioned that the dosages of DOCA or cortisone used were adequate to suppress adrenocortical regeneration in almost all the animals. Curiously, the one animal that developed a

carcinoma, even though given DOCA, was the only animal in the group of 7 which had regenerated adrenocortical tissue. This would seem to be mere coincidence, particularly when the results with this animal are compared to those with the group given 2 mg of cortisone in which 8 of the 9 animals developing tumors failed to regenerate adrenocortical tissue.

The protective effect of DOCA in these experiments is striking, but the mechanism of its action is not clear. One might postulate that it causes a decreased permeability of the liver-cell membrane, which decreases the amount of carcinogen entering the cell and therefore the development of liver cancer. Kotin (*31*) has shown that estrogen decreases the number of carbon particles that enter the liver cell as it decreases the permeability of the cell membrane, and he noted some protective effect against hepatic carcinogenesis by estrogens. Or, one might postulate some competitive or blocking effect comparable to the preferential tight binding of C^{14}-labeled estradiol by rat-liver protein in homogenates as demonstrated by Riegel and Mueller (*32*).

Probably the most striking finding derived from these experiments is the demonstration of the important procarcinogenic effect of anabolic hormones. It would seem that this is probably dependent on their effect on protein synthesis by liver cells. The Millers (*33*, *34*) have shown that 4-dimethylaminoazobenzene and a number of its derivatives are bound to protein in the hepatic cell. Weisburger *et al.* (*35*) demonstrated that radioactivity from labeled *N*-2-fluorenylacetamide is similarly bound by protein of liver cells. Although it has not been specifically demonstrated that *N*-2-fluorenyldiacetamide is bound to protein, it is assumed that its behavior is entirely similar to *N*-2-fluorenylacetamide in this respect.

Gelboin and the Millers (*36*) found that the carcinogens are bound to protein currently being formed. Thus, the finding that delay in administration of testosterone to castrated females completely prevented cirrhosis and carcinoma takes on added significance and further emphasizes the importance of the rate of protein anabolism at the time the carcinogen is given.

A careful analysis by Farber *et al.* (*37*, *38*) of the effects of ethionine (a hepatic carcinogen) on protein synthesis and the production of fatty liver in rats revealed a distinct hormonal influence. These findings closely parallel the hormonal relationships in hepatic carcinogenesis of the present experiments and those of others (*10–13*). Male rats, castrated males given testosterone, and castrated females given testosterone (*37*) or other anabolic hormones (*38*) were protected against fatty livers when given ethionine. Growth hormone and thyroxine had some protective effect, but intact and castrated females and castrated males were not protected. Administration of estradiol, deoxycorticosterone, thyrotrophic hormone, prolactin, and crystalline zinc insulin were not protective. Cortisone and adrenocorticotrophic hormone actually intensified the lesion (*38*). Ranney and Drill (*39*) confirmed the protective effect of norethandrolone

and showed that progesterone was ineffective. The effect of adrenalectomy apparently has not been studied. From this, one can conclude that even blocking of protein synthesis by ethionine can be overcome by hormones, particularly androgenic or anabolic hormones.

The recent work of Kochakian (*40*) suggests that androgens act by altering cellular enzyme activity. In rat liver he showed that the activity of several enzymes concerned with protein metabolism including aspartic-glutamic transaminase, alanine-glutamic transaminase, and *d*-amino acid oxidase are diminished by castration and restored by testosterone. Thus it would seem likely that androgenic and probably anabolic hormones have their procarcinogenic and procirrhotic effects on the rat liver through their fundamental influence over intracellular enzymes concerned with protein metabolism. In the case of carcinogenesis this means increased protein synthesis and increased carcinogen binding.

It is clear, however, that carcinogen binding represents only the first step in carcinogenesis because, over periods ranging from weeks to months after detectable carcinogen has disappeared (*41*) and several liver-cell generations later, abnormal hyperplastic areas, nodules, and finally tumors appear. This indicates that a disturbance was introduced into some of the liver cells which was "remembered" long after the carcinogen had disappeared and that it had been passed on to the new generations of liver cells.

It has been shown in studies of protein synthesis that labeled amino acids are incorporated early in the microsomal fraction of rat liver (*42–44*). The bound carcinogen is likewise found principally in the ribonucleoprotein containing microsomal fraction of the liver cells (*35*, *45*, *46*). The work of the Millers (*33*) implies that the carcinogen may be actually incorporated into the newly formed protein and that as a result an abnormal "protein" is formed. If the carcinogen is bound into the ribonucleoproteins it can serve as a template for the subsequent formation of abnormal protein devoid of carcinogen. This nucleoprotein, known to be capable of self-replication, provides an excellent "memory" apparatus for the continued formation of abnormal protein from a template previously made from carcinogen-containing protein (*47*, *48*). Further, the studies of Stent (*49*) suggest that a single modified strand of ribonucleoprotein may serve as a template for the formation of new deoxyribonucleoprotein. This provides a method of deoxyribonucleoprotein alteration and production of a heritable induced defect simply by alteration of a single strand of ribonucleoprotein by the carcinogen.

One is readily reminded of the analogy of this hypothesis to the action of viruses which induce tumors in animals and become intimately involved in the deoxyribonucleoprotein and ribonucleoprotein synthetic processes of cells. The viral genome perpetuates itself by substituting itself for the normal nucleoprotein of the same kind and abnormal protein is thereby synthesized (*50*).

One might also suggest that various types of radiation may act in a similar manner on the heritable nucleoprotein of the cells in producing

he abnormal nucleoprotein which at some later time becomes manifest in n uncontrolled, rapidly growing colony of cells we call cancer.

APPENDIX

Scoring Procedure

To represent adequately the differences between the various groups of animals, it was necessary to attempt conversion of the several pathological changes to some numerical value. No such arbitrary conversion could hope to satisfy even a majority of investigators, but an educated relative evaluation is probably better than simple incidence figures when so many other parameters are available. The general formula can be written $(A + B + C)\ D =$ score per animal.

A. The following values were assigned to the various lesions that developed with increasing weight according to the importance of the lesions. Only the most advanced lesion was used in scoring this section.
A = Most advanced lesion

	Number of well-developed hepatomas					
	0	1	2	3	4	>4
Normal liver	0	—	—	—	—	—
Foci or areas of hyperplasia	5	—	—	—	—	—
Nodules of hyperplasia	10	—	—	—	—	—
Small hepatoma	25	—	—	—	—	—
Well-developed hepatoma	—	50	60	70	80	100

B. To the figure thus arrived at was added an additional sum for metastases. As can be seen, the premium awarded for metastases reflects the authors' healthy respect for this aspect of malignant growths.
B = Metastases

None	0
Rare	100
Few	150
Many	200
Massive	250

C. Then a product derived from the weight of the liver in grams times one plus the excess diameter over 1 cm of the largest tumor was divided by 2 and the quotient added to the subtotal of the above. This takes into account the total mass of tissue in the liver and gives special emphasis to tumors more than 1 cm in diameter. It also diminishes the importance of enlargement due to multiple small lesions including lesions that have

not yet reached a definitely malignant state and returns such enlargement to a more nearly normal weight.

$$C = \frac{\text{Weight of liver} \times (1 + \text{excess of diameter over 1 cm})}{2}$$

D. Finally, to correct for survival time the total score was multiplied by a survival factor which ranged from 1 for 42 weeks or more to 2 for 25 weeks, with straight-line conversion for all intermediate survival times. All animals not surviving the minimal latent period of 25 weeks were not included in the experiments. All animals surviving more than 42 weeks were included with the 42-week group for calculation of the survival factor.

$$D = \textit{Survival factor} = 1 + \frac{42 - \text{number of weeks up to 42}}{17}$$

REFERENCES

(*1*) Edmondson, H. A., and Steiner, P. E.: Primary carcinoma of the liver; study of 100 cases among 48,900 necropsies. Cancer 7: 462–503, 1954.

(*2*) Davies, J. N. P.: Human implications. Primary carcinoma of the liver in Africans. J. Nat. Cancer Inst. Supp. 15: 1637–1644, 1955.

(*3*) Bielschowsky, F.: Distant tumours produced by 2-amino- and 2-acetyl-aminofluorene. Brit. J. Exper. Path. 25: 1–4, 1944.

(*4*) Stasney, J., Paschkis, K. E., Cantarow, A., and Rothenberg, M. S.: Neoplasms in rats with 2-acetylaminofluorene and sex hormones. Cancer Res. 7: 356–362, 1947.

(*5*) Kirby, A. H. M.: The combined action of 2-acetylaminofluorene and sex hormones in the Wistar rat. Brit. J. Cancer 1: 68–79, 1947.

(*6*) Symeonidis, A.: Tumors induced by 2-acetylaminofluorene in virgin and breeding females of five strains of rats and in their offspring. J. Nat. Cancer Inst. 15: 539–549, 1954.

(*7*) Leathem, J. H.: Influence of sex on 2-acetylaminofluorene-induced liver tumors in rats and mice. (Abstract.) Cancer Res. 11: 266, 1951.

(*8*) Rumsfeld, H. W., Jr., Miller, W. L., Jr., and Baumann, C. A.: A sex difference in the development of liver tumors in rats fed 3′-methyl-4-dimethylaminoazobenzene or 4′-fluoro-4-dimethylaminoazobenzene. Cancer Res. 11: 814–819, 1951.

(*9*) Morris, H. P., and Firminger, H. I.: Influence of sex and sex hormones on development of hepatomas and other hepatic lesions in strain A × C rats ingesting 2-diacetylaminofluorene. J. Nat. Cancer Inst. 16: 927–949, 1956.

(*10*) Bielschowsky, F., and Hall, W. H.: Carcinogenesis in the thyroidectomized rat. Brit. J. Cancer 7: 358–366, 1953.

(*11*) Robertson, C. H., O'Neal, M. A., Griffin, A. C., and Richardson, H. L.: Pituitary and adrenal factors involved in azo dye liver carcinogenesis. Cancer Res. 13: 776–779, 1953.

(*12*) Robertson, C. H., O'Neal, M. A., Richardson, H. L., and Griffin, A. C.: Further observations on the role of the pituitary and the adrenal gland in azo dye carcinogenesis. Cancer Res. 14: 549–553, 1954.

(*13*) Griffin, A. C., Richardson, H. L., Robertson, C. H., O'Neal, M. A., and Spain, J. D.: The role of hormones in liver carcinogenesis. J. Nat. Cancer Inst. Supp. 15: 1623–1632, 1955.

(*14*) Morris, H. P., and Firminger, H. I. Unpublished data.

(16) Skoryna, S. C.: Factors affecting liver carcinogenesis by 2-acetyl-aminofluorene. Proc. Canad. Cancer Res. Conf. 1: 107–113, 1954.

(17) Symeonidis, A., Mulay, A. S. and Burgoyne, F. H.: Effect of adrenalectomy and of desoxycorticosterone acetate on the formation of liver lesions in rats fed *p*-dimethylaminoazobenzene. J. Nat. Cancer Inst. 14: 805–817, 1954.

(18) Eversole, W. J.: Inhibition of azo dye carcinogenesis by adrenalectomy and treatment with desoxycorticosterone trimethylacetate. Proc. Soc. Exper. Biol. & Med. 96: 643–646, 1957.

(19) Da Vanzo, J. P., and Eversole, W. J.: The effect of adrenalectomy and deoxycorticosterones on liver enzymes and protein in rats fed 3′-methyl-4-dimethylaminoazobenzene. Cancer Res. 18: 796–801, 1958.

(20) Morris, H. P., Dubnik, C. S., and Johnson, J. M.: Studies on the carcinogenic action in the rat of 2-nitro-, 2-amino-, 2-acetylamino-, and 2-diacetylaminofluorene after ingestion and after painting. J. Nat. Cancer Inst. 10: 1201–1213, 1950.

(21) Kinosita, R.: Studies on the cancerogenic chemical substances. Tr. Soc. path. jap. 27: 665–727, 1937.

(22) Orr, J. W.: The histology of the rat's liver during the course of carcinogenesis by butter-yellow (*p*-dimethylaminoazobenzene). J. Path. & Bact. 50: 393–408, 1940.

(23) Firminger, H. I.: Histopathology of carcinogenesis and tumors of the liver in rats. J. Nat. Cancer Inst. Supp. 15: 1427–1442, 1955.

(24) Kinosita, R.: Studies on the cancerogenic azo and related compounds. Yale J. Biol. & Med. 12: 287–300, 1940.

(25) Opie, E. L.: The pathogenesis of tumors of the liver produced by butter yellow. J. Exper. Med. 80: 231–246, 1944.

(26) Mulay, A. S., and Firminger, H. I.: Liver tumors induced in rats by *p*-dimethylaminobenzene-1-azo-1-naphthalene compared with tumors induced by *p*-dimethylaminoazobenzene. J. Nat. Cancer Inst. 13: 35–55, 1952.

(27) Stewart, H. L., and Snell, K. C.: The histopathology of experimental tumors of the liver of the rat. Acta Unio internat. contra cancrum 8: 770–803, 1957.

(28) Saunders, F. J., and Drill, V. A.: Comparative androgenic and anabolic effects of several steroids. Proc. Soc. Exper. Biol. & Med. 94: 646–649, 1957.

(29) Reuber, M. D.: Endometrial sarcomas of the uterus and carcinosarcomas of the submaxillary salivary gland in castrated A × C strain female rats receiving *N*-2-fluorenyldiacetamide and norethandrolone. J. Nat. Cancer Inst. 25: 1141–1153, 1960.

(30) Sidransky, H., Wagner, B. P., and Morris, H. P.: Sex difference in liver tumorigenesis in rats ingesting *N*-2-fluorenylacetamide. J. Nat. Cancer Inst. 26: 151–187, 1961.

(31) Reuber, M. D., and Firminger, H. I.: Unpublished data.

(32) Kotin, P.: Personal communication.

(33) Riegel, I. L., and Mueller, G. C.: Formation of a protein-bound metabolite of estradiol-16-C^{14} by rat liver homogenates. J. Biol. Chem. 210: 249–257, 1954.

(34) Miller, E. C., and Miller, J. A.: Biochemical investigations on hepatic carcinogenesis. J. Nat. Cancer Inst. Supp. 15: 1571–1590, 1955.

(35) ———: The presence and significance of bound aminoazo dyes in the livers of rats fed *p*-dimethylaminoazobenzene. Cancer Res. 7: 468–480, 1947.

(36) Weisburger, E. K., Weisburger, J. H., and Morris, H. P.: Studies on the metabolism of 2-acetylaminofluorene-9-C^{14}. Arch. Biochem. 43: 474–484, 1953.

(37) Gelboin, H. V., Miller, J. A., and Miller, E. C.: Studies on hepatic protein-bound dye formation in rats given single large doses of 3′-methyl-4-dimethylaminoazobenzene. Cancer Res. 18: 608–617, 1958.

(38) Farber, E.: Studies on the chemical pathology of lesions produced by ethionine. Arch. Path. 67: 1–8, 1959.

(*38*) FARBER, E., and SEGALOFF, A.: Effect of androgens and growth and other hormones on ethionine fatty liver in rats. J. Biol. Chem. 216: 471–477, 1955.

(*39*) RANNEY, R. E., and DRILL, V. A.: The ability of 17-ethyl-19-nortestosterone to block ethionine-induced fatty liver in rats. Endocrinology. 61: 476–477, 1957.

(*40*) KOCHAKIAN, C. D.: Mechanisms of androgen actions. Lab. Invest. 8: 538–556, 1959.

(*41*) MILLER, E. C., and MILLER, J. A.: Symposium on immunogenetics and carcinogenesis; *in vivo* combinations between carcinogens and tissue constituents and their possible role in carcinogenesis. Cancer Res. 12: 547–556, 1952.

(*42*) LITTLEFIELD, J. W., KELLER, E. B., GROSS, J., and ZAMECNIK, P. C.: Studies on cytoplasmic ribonucleoprotein particles from the liver of the rat. J. Biol. Chem. 217: 111–124, 1955.

(*43*) BOORSOOK, H., DEASY, C. L., HAAGEN-SMIT, A. J., KEIGHLEY, G., and LOWRY, P. H.: Metabolism of C^{14}-labeled glycine, L-histidine, L-leucine, and L-lysine. J. Biol. Chem. 187: 839–848, 1950.

(*44*) LITTLEFIELD, J. W., and KELLER, E. B.: Incorporation of C^{14} amino acids into ribonucleoprotein particles from the Ehrlich mouse ascites tumor. J. Biol. Chem. 224: 13–30, 1957.

(*45*) PRICE, J. M., MILLER, E. C., MILLER, J. A., and WEBER, G. M.: Studies on the intracellular composition of livers from rats fed various aminoazo dyes. II. 3′-methyl-, 2′-methyl-, and 2-methyl-4-dimethylamino-azobenzene, 3-methyl-4-monomethylaminoazobenzene, and 4′-fluoro-4-dimethylaminoazobenzene. Cancer Res. 10: 18–27, 1950.

(*46*) PRICE, J. M., MILLER, E. C., and MILLER, J. A.: The intracellular distribution of protein, nucleic acids, riboflavin, and protein-bound amino azo dye in the livers of rats fed *p*-dimethylaminoazobenzene. J. Biol. Chem. 173: 345–353, 1948.

(*47*) Microsomal Particles and Protein Synthesis. First Symposium Biophysical Society. New York, Pergamon Press, 1958.

(*48*) COHEN, S. S.: A hypothesis on a possible competitive relation between DNA synthesis and protein synthesis. Cancer Res. 20: 698–699, 1960.

(*49*) STENT, G. S.: Mating in the reproduction of bacterial viruses. Advances Virus Res. 5: 95–149, 1958.

(*50*) DULBECCO, R.: A consideration of virus-host relationship in virus-induced neoplasia at the cellular level. Cancer Res. 20: 751–761, 1960.

PLATES

PLATE 67

FIGURE 1.—*Cirrhosis.* Proliferation of small bile ducts, as well as an increase in larger bile ducts, in portal areas. Periportal liver cells have larger nuclei and basophilic staining cytoplasm. Hematoxylin and eosin. × 154

FIGURE 2.—*Cirrhosis.* Proliferation of small bile ducts extends into adjacent lobules and isolates hepatic parenchymal cells. Liver cells, which have large, darkly staining nuclei and increased cytoplasm, represent foci of hyperplasia. Hematoxylin and eosin. × 207

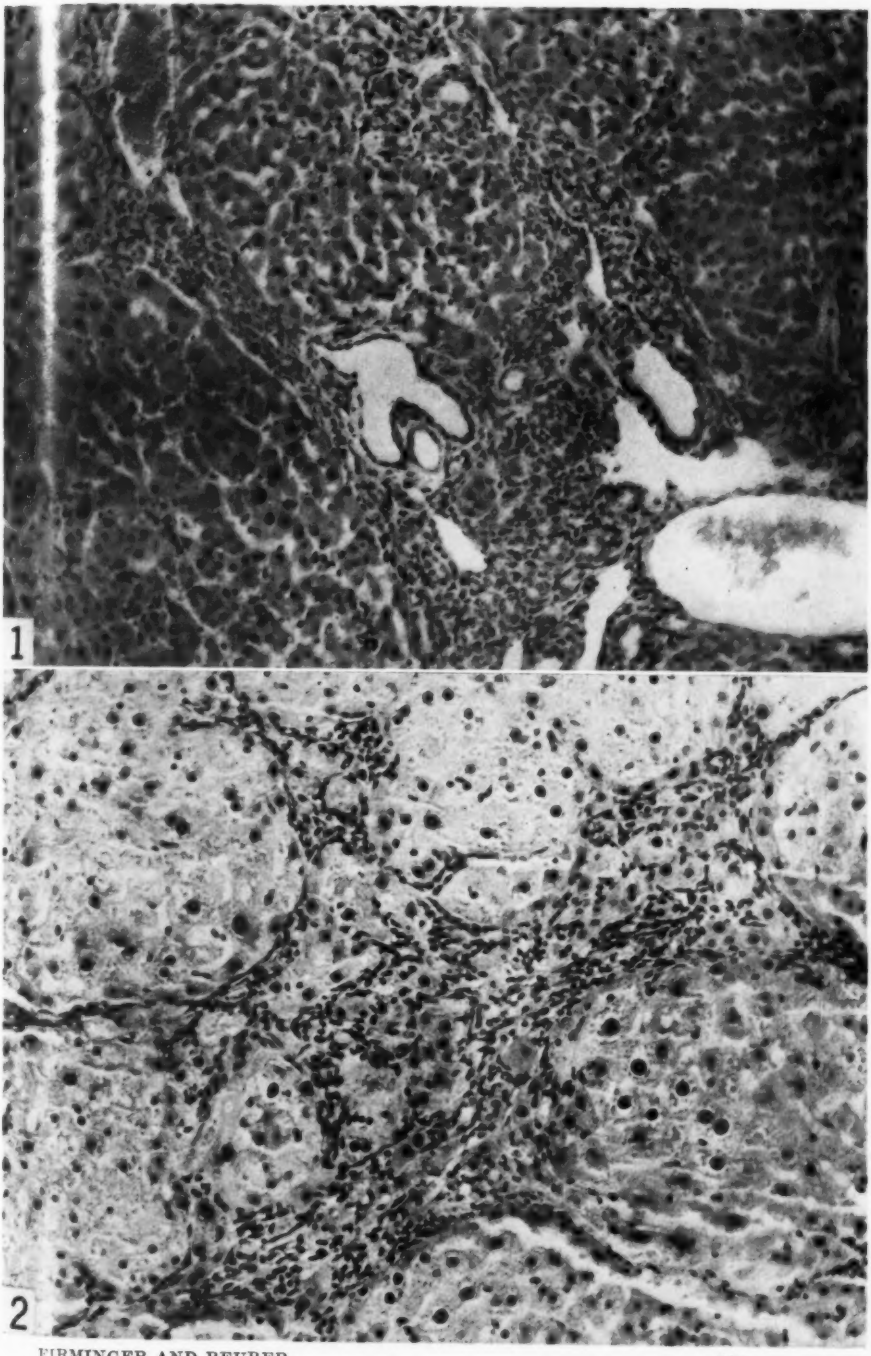

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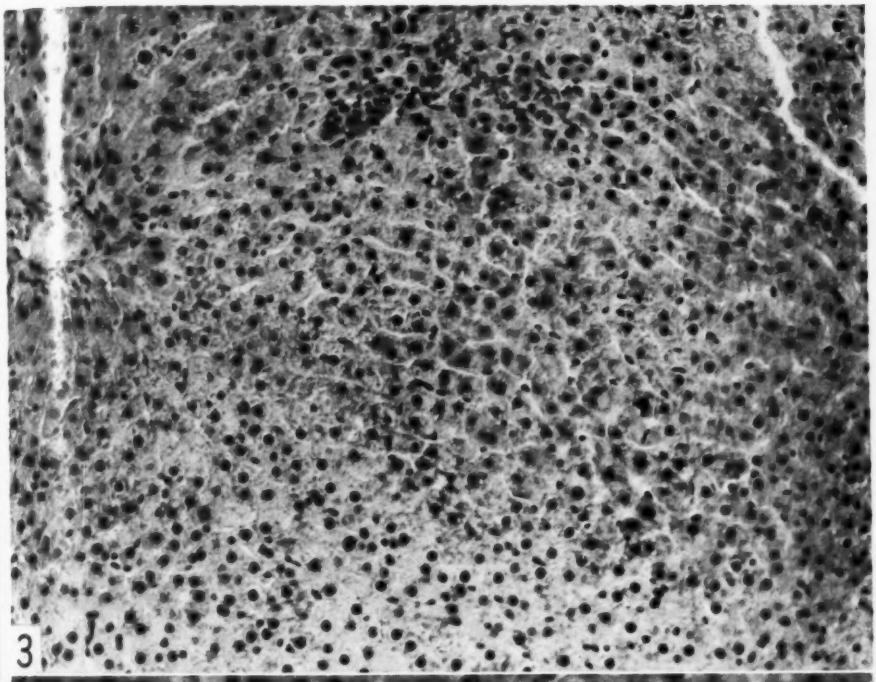
PLATE 68

FIGURE 3.—*Focus of hyperplasia.* Groups of cells with larger nuclei, more abundant cytoplasm with basophilic material, and frequently double nuclei. They are not clearly demarcated from the uninvolved surrounding parenchyma. Hematoxylin and eosin. × 207

FIGURE 4.—*Focus of hyperplasia.* *Arrows* point to a focus of hyperplastic liver cells. Hematoxylin and eosin. × 415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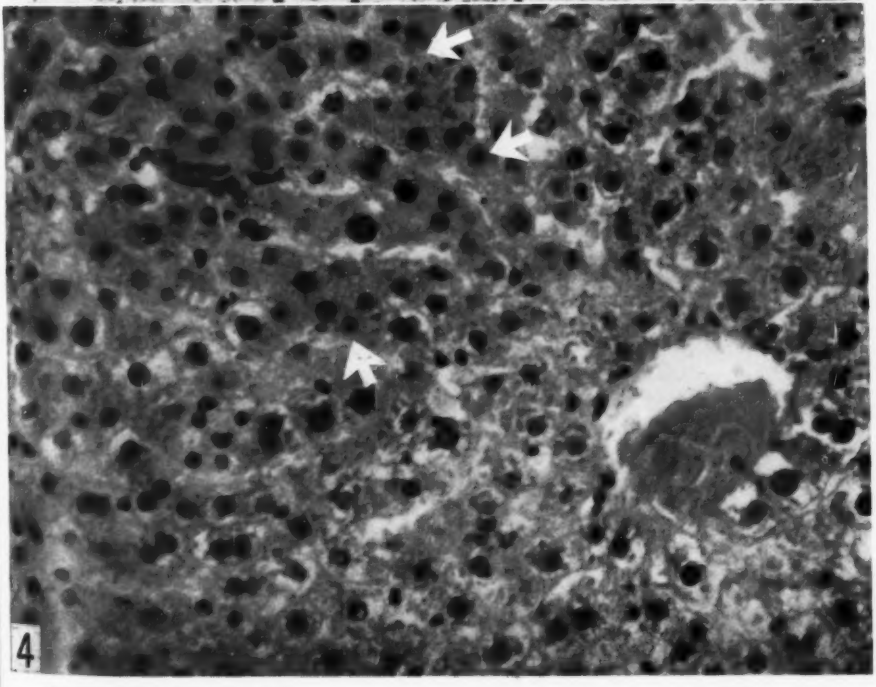
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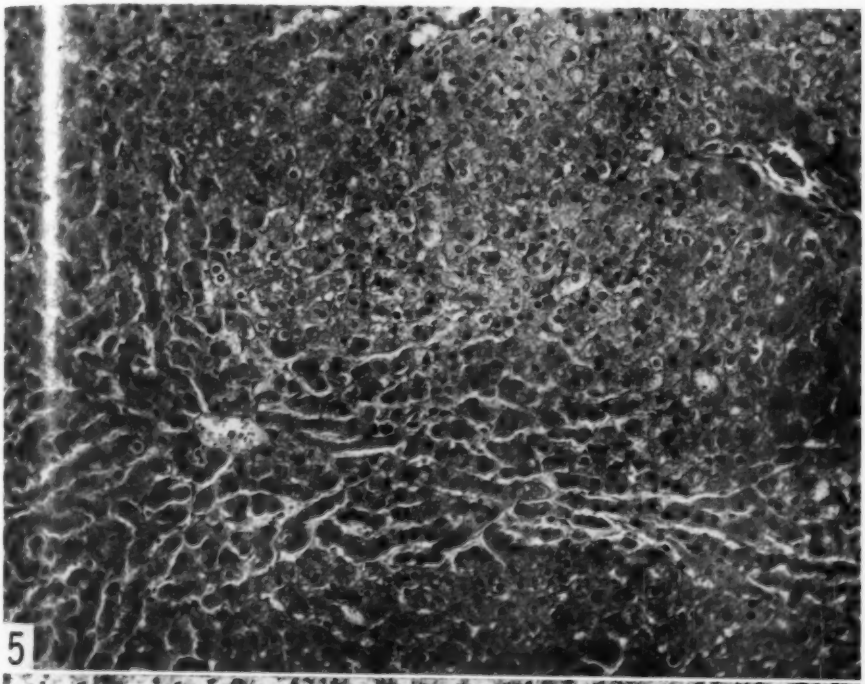
PLATE 69

FIGURE 5.—*Areas of hyperplasia.* Portal areas near *top* and to *right.* Adjacent to the portal areas there is an increased number of cells, which are small, closely packed, and contain basophilic material in the cytoplasm. Similar periportal hyperplasia can be seen extending up from the *bottom* on *right.* Hematoxylin and eosin. × 156

FIGURE 6.—*Area of hyperplasia.* The focus of hyperplasia increases in size until it becomes an area which is well demarcated from the surrounding liver. Area of hyperplasia occupies *upper right corner* of photomicrograph. Hematoxylin and eosin. × 207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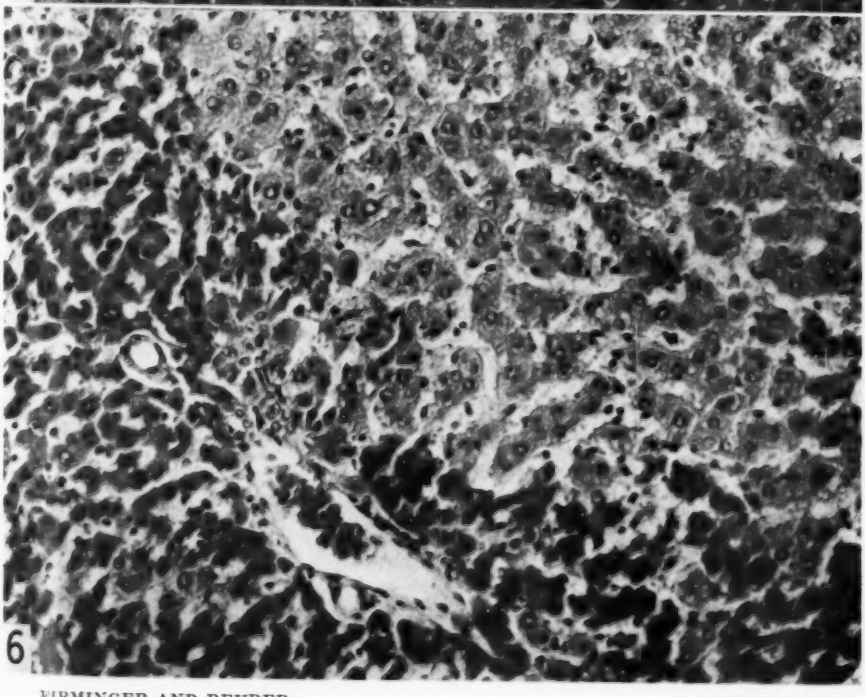
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PLATE 70

FIGURE 7.—*Nodule of hyperplasia.* Hepatic cords are less orderly and adjacent hepatic parenchyma is compressed and distorted by spherical growth. Hematoxylin and eosin. × 154

FIGURE 8.—*Nodule of hyperplasia.* There is less well-defined cord structure compared to figure 7. Hematoxylin and eosin. × 207

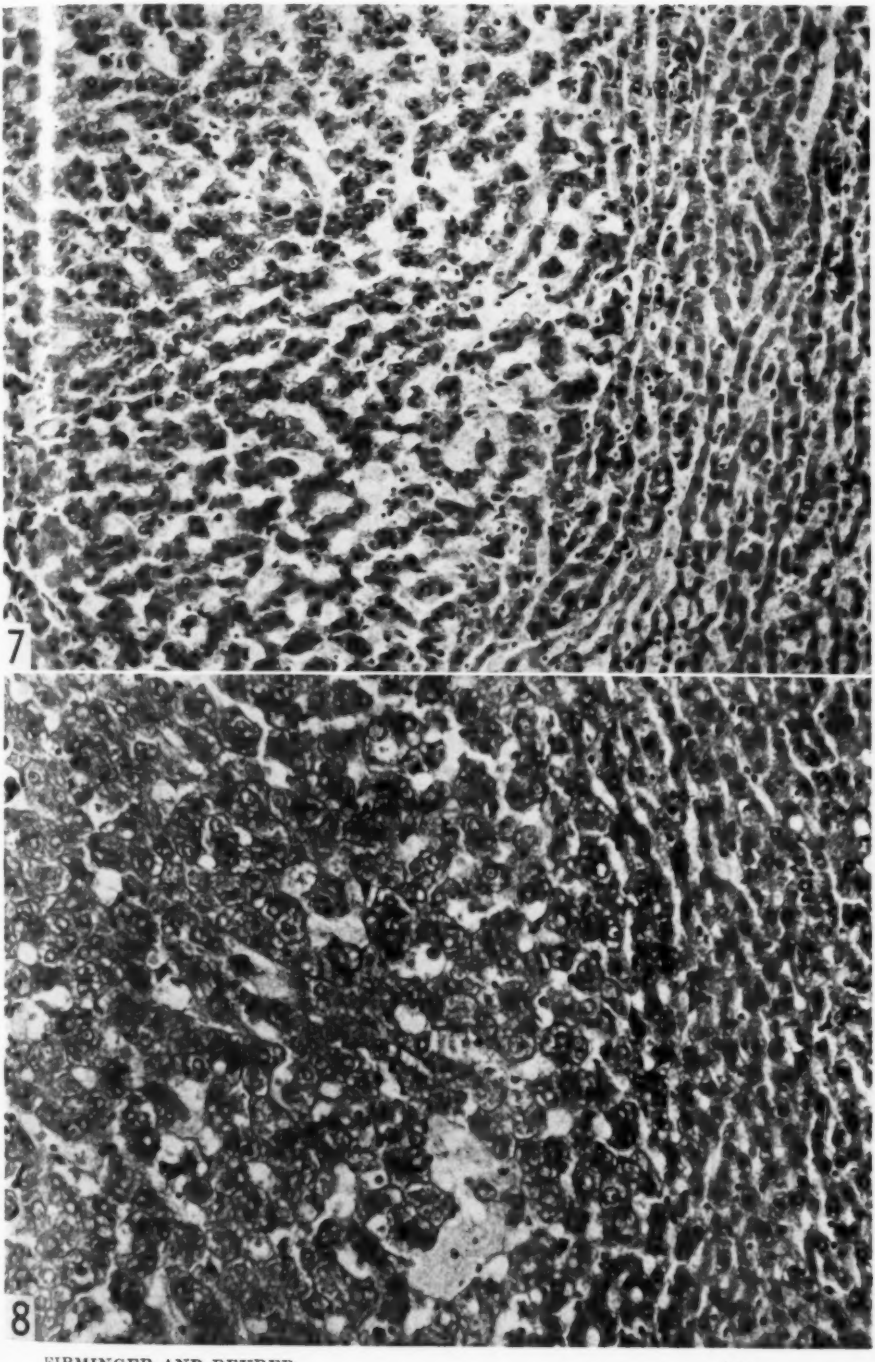
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PLATE 71

FIGURE 9.—*Nodule of hyperplasia with atypicality.* Cord structure is distorted and irregular. Hyperplastic cells vary in size and nuclei tend to be larger and more vesicular and sometimes vary in staining. There is compression of surrounding liver tissue. Hematoxylin and eosin. × 154

FIGURE 10.—*Small hepatoma.* Cells are large and atypical with variability in size, shape, and staining. Nuclei are large and vesicular with prominent enlarged nucleoli. There are mitotic figures (not shown) and foci of degeneration and necrosis. Adjacent parenchyma is compressed and distorted. Hematoxylin and eosin. × 154

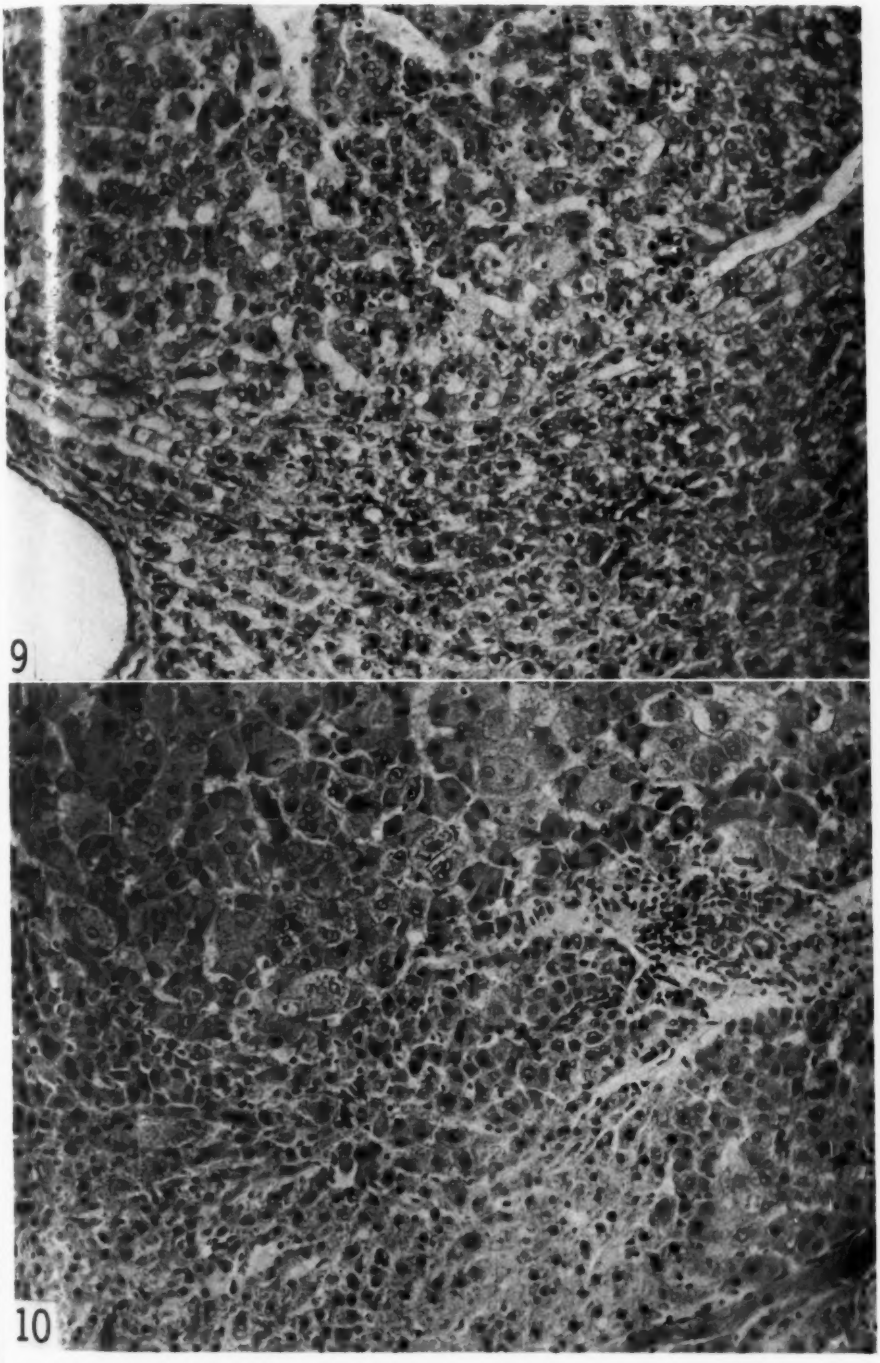
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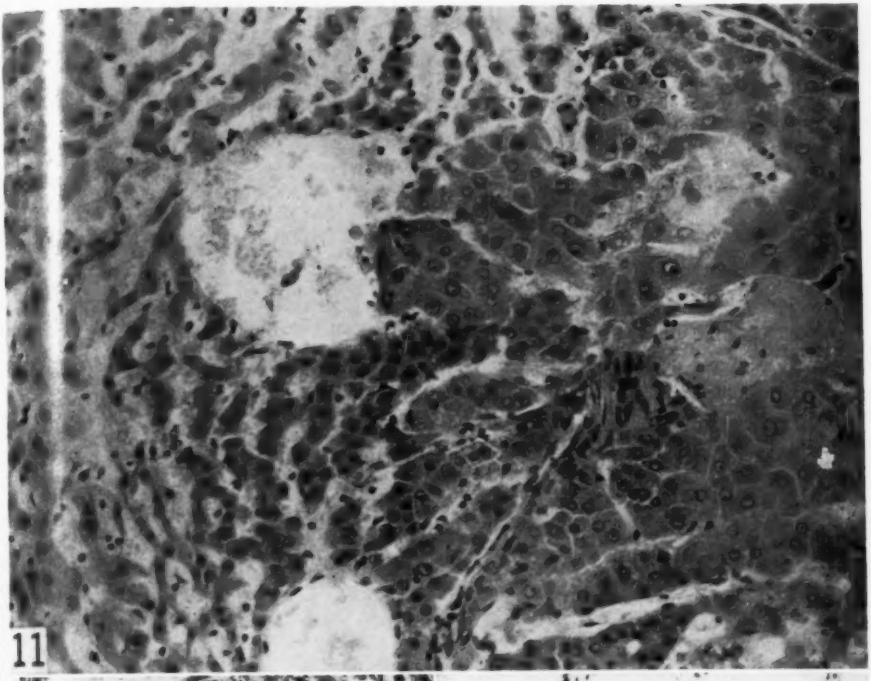
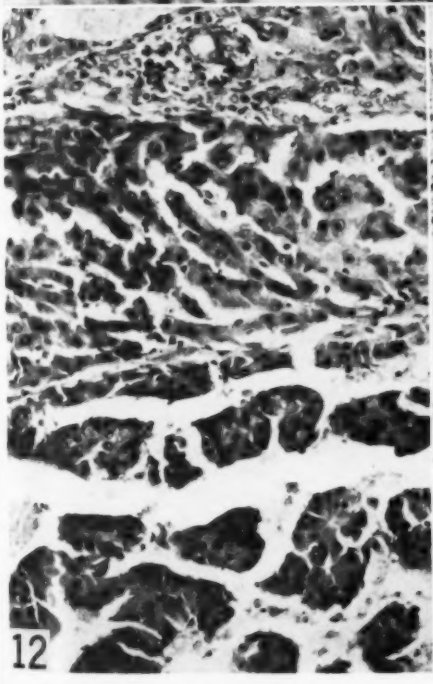
PLATE 72

FIGURE 11.—*Well-differentiated hepatocellular carcinoma with invasion of the portal vein.* Tumor cells resemble hepatic parenchymal cells with large round or oval nuclei, prominent nucleoli, marginal chromatin lining the nuclear membrane, and abundant eosinophilic or, at times, basophilic cytoplasm. Hematoxylin and eosin. × 207

FIGURE 12.—*Well-differentiated hepatocellular carcinoma.* In *center* the tumor is arranged in cords. On *bottom* the cells are arranged in islands and trabeculae. Hematoxylin and eosin. × 154

FIGURE 13.—*Well-differentiated hepatocellular carcinoma, metastatic to the lung.* Tumor cells resemble liver cells. Hematoxylin and eosin. × 154





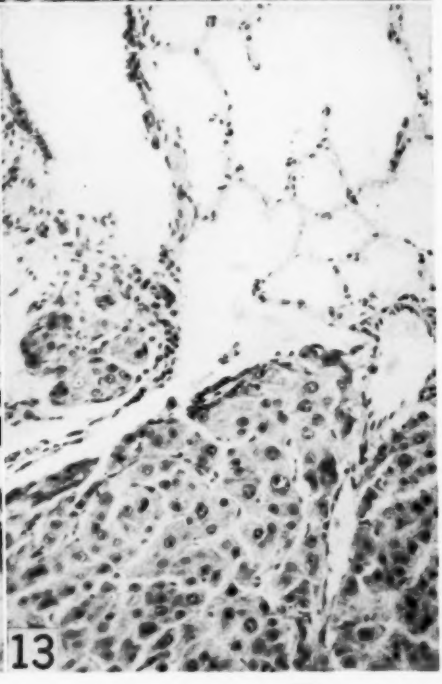


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PLATE 73

FIGURE 14.—*Cholangiomatous area of cholangiohepatoma.* Columnar bile-duct epithelial cells with pale cytoplasm, scalloped borders, and basal nuclei without nucleoli form ductlike structures. Hematoxylin and eosin. × 207

FIGURE 15.—*Anaplastic hepatocellular carcinoma.* Cells at *top* and *center* have varying sizes and shapes. Their nuclei are crowded and vary in size and staining. The cytoplasm is basophilic. Hematoxylin and eosin. × 207

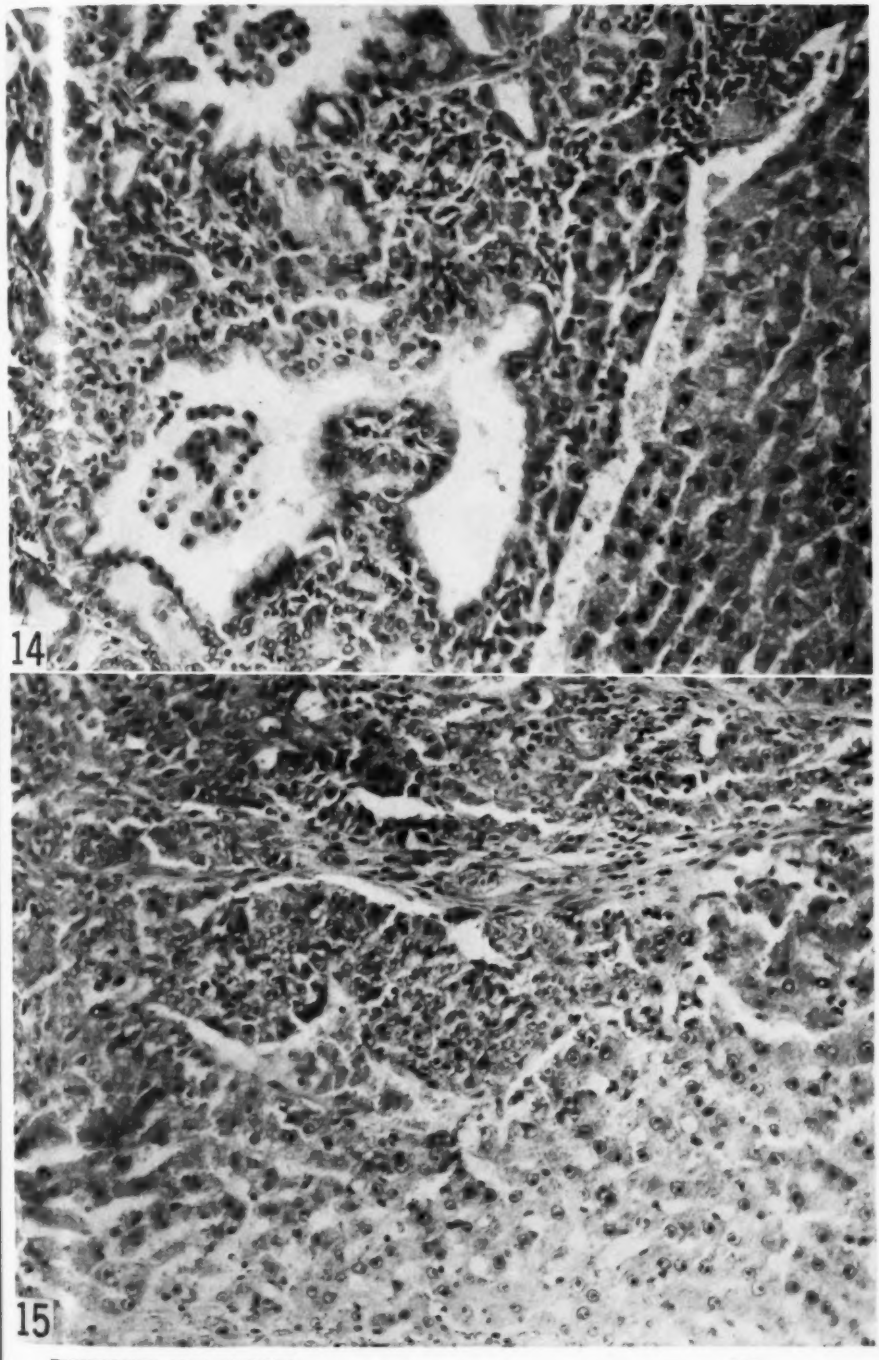
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PLATE 74

FIGURE 16.—*Intact male.* A tan, umbilicated hepatocellular carcinoma 2 × 3 cm in diameter in the median lobe of the liver. There are also many cysts, as well as cirrhosis. Liver weighed 19.6 g.

FIGURE 17.—*Castrated male.* A dark, purple, smooth hepatocellular carcinoma 1.5 × 2.5 cm in diameter also in the median lobe. There are few cysts, and cirrhosis of the liver is absent. Liver weighed 17.6 g. This was the largest tumor in the castrated group.

FIGURE 18.—*Intact female receiving testosterone.* A 1 cm gray-white hepatocellular carcinoma in median lobe of liver. There are both cirrhosis and multiple small cysts. This is similar to the findings in females given norethandrolone and to those in intact males receiving norethandrolone or testosterone, except that the tumors in all of these latter groups were slightly larger. Liver weighed 14.5 g.

FIGURE 19.—*Intact female.* Liver surface is smooth with only a few scattered small cysts. Livers of castrated females, castrated and adrenalectomized females, and of adrenalectomized females were similar. Liver weighed 9.9 g.

FIRMINGER AND REUBER

PLATE 75

FIGURE 20.—*Adrenalectomized male.* Large areas of liver are replaced by gray-white hepatocellular carcinoma. Tumor mass on *right* measures 6.5 cm in greatest dimension. Centrally there is necrosis and hemorrhage. There are scattered small cysts and cirrhosis is present but not well shown. Liver weighed 64.5 g. There were similar findings for adrenalectomized females given testosterone and castrated males given testosterone.

FIGURE 21.—*Adrenalectomized male.* Innumerable metastases replacing most of both lungs.

FIGURE 22.—*Adrenalectomized male given deoxycorticosterone.* Surfaces are smooth and there are no cysts. Liver resembles that of normal control rats. Liver weighed 8.7 g.

FIGURE 23.—*Castrated male given norethandrolone.* Three main tumor masses; largest of the two shown measures 2.5 cm in diameter. Cirrhosis and cysts are present. Liver weighed 48.6 g. This is also representative of adrenalectomized, castrated females given testosterone and adrenalectomized males receiving cortisone (2 mg dose).

FIRMINGER AND REUBER

Glucose Metabolism of Tumors Induced by Rous Sarcoma Virus. I. Comparison of Chorio-allantoic Membrane With Tumor of the Chorio-allantois Induced by Rous Sarcoma Virus [1, 2]

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SUMMARY

Essentially linear rates of glucose utilization were observed over various concentrations: (*a*) Tumor of the chorio-allantois induced by Rous sarcoma virus (RSV tumor) had a maximal rate of glucose utilization 6 times that of chorio-allantoic membrane (CAM). (*b*) Half maximal velocity was achieved with 5 mmoles per liter glucose by RSV tumor and 1.3 mmoles per liter glucose by CAM. Glycolytic activity was markedly greater with RSV tumor than CAM under aerobic conditions. Under anaerobic conditions the difference was not as pronounced: (*a*) Lactate accounted for 50 to 100 percent of C^{14} glucose metabolized by RSV tumor under both aerobic and anaerobic conditions. (*b*) About 25 percent of the glucose metabolized by CAM aerobically was recovered as lactate; anaerobically, 30 to 80 percent. With lower concentrations of glucose a greater percentage could be considered lactate from both tissues. Only 5 to 6 percent of the glucose used by RSV tumor was recovered as CO_2, while there was 50 to 60 percent from CAM. About 5 percent of the glucose used by RSV tumor was recovered in fat. This was 10 to 20 times the amount recovered as glycogen. Very little C^{14} glucose was recovered as glycogen or fatty acids in CAM. Lactate selectively suppressed oxygen uptake of RSV tumor. Proposed mechanisms of the Crabtree effect are discussed with regard to the data presented.—J. Nat. Cancer Inst. 27: 597–609, 1961.

THE CLASSIC studies of Burk *et al.* (*1*), concerning a variety of transplantable chicken tumors and the filterable Rous chicken-tumor 1 (*2*), were the first comprehensive metabolic studies of chicken tumors. These investigators grew Rous chicken-tumor 1 by inoculating fresh chicken-tumor pulp onto the chorio-allantoic membrane (CAM) of embryonated chicken eggs. To describe further metabolic changes associated with neoplastic growth a system of induction was sought that would rapidly yield tumors from a tissue and could be readily reproduced upon demand. Preparations of standard lots of Rous sarcoma virus (RSV) by Dr. W. Ray Bryan, of the National Cancer Institute, give uniform induction when inoculated onto dropped CAM's of eggs from flocks of susceptible

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[2] This work was supported in part by research grant C-4692 of the National Cancer Institute, National Institutes of Health, Public Health Service, and grant E-212 of the American Cancer Society, Inc.

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chickens. Under these conditions metabolic parameters of induced tumors and tissue of origin can be studied. Further, since virus uniformly transforms susceptible cells, the sequential metabolic changes associated with induction of neoplastic growth can eventually be approached.

In previous studies from these laboratories (*3–7*), RSV-induced tumors of the CAM (RSV tumor) of chicken eggs provided virus-transformed tissue that could be compared with uninfected tissue of origin. The following report compares the glucose metabolism of RSV tumors, 6 to 7 days after infection, with that of uninfected CAM.

One of the most striking differences in metabolism between the RSV tumor and CAM is the effect of glucose on tissue respiration. RSV tumor incubated with added glucose exhibits a marked depression in oxygen consumption. Crabtree (*8*) first described the effect of glucose on the respiration of tumor tissue, and this phenomenon is known as the Crabtree effect. Previous work established that various concentrations of glucose can induce the Crabtree effect in Rous sarcoma (*1, 4, 6*). Within certain limits, the degree of depression of oxygen consumption depended on the initial glucose concentration in the incubation medium. To characterize better the metabolic differences between RSV tumor and CAM, as well as to investigate further metabolic factors that might contribute to the Crabtree effect, the experiments described were designed to: 1) characterize the rates of glucose metabolism in the normal and neoplastic tissues, 2) account for the major metabolic products of glucose metabolism in both tissues, and 3) examine the effect of products of glucose metabolism on the induction of the Crabtree effect.

MATERIALS AND METHODS

Virus.—Standard RSV Lot #842, provided through the courtesy of Dr. W. Ray Bryan, National Cancer Institute, was used in all experiments to induce tumors of the CAM. A dose of $10^{5.5}$ tumor-forming units (*9, 10*) was the standard inoculum. The diluent for virus was cold saline plus 2 percent inactivated horse serum. A volume of 0.2 ml was inoculated onto the dropped CAM of 9- to 12-day-old White Leghorn eggs. RSV-inoculated and control eggs were incubated at 37° C.

Tissue.—Tumors were routinely harvested 6 to 7 days after inoculation. RSV tumors were excised from surrounding CAM and sliced with a Stadie-Riggs hand microtome. Normal CAM from control eggs was cut into pieces of approximately 1 cm^2. All tissue was washed thoroughly in saline, blotted on filter paper for removal of excess fluid, rapidly weighed on a Roller Smith torsion balance, and placed directly into the incubation medium. In most experiments RSV tumor or CAM was excised from 16-day-old eggs. It is significant that unpublished data of Dr. George Weber comparing the activities of glycolytic enzymes of CAM from eggs 10, 13, or 16 days old do not show marked differences.

Metabolic Studies

Manometry.—Oxygen uptake was determined by the direct method of Warburg. Air was the gas phase for aerobic experiments. A medium volume of 2 ml was used in all manometric experiments. An incubation temperature of 37° C and a standard equilibration time of 10 minutes were used. Readings were made at hourly intervals for 4 hours. Approximately 200 mg wet tissue was incubated in the 2 ml volume of medium. The weight of the tissue of each flask, after drying overnight at 80° C, was determined. All data were plotted as average of triplicate results.

Medium and substrates.—The medium for manometric studies was the following modification of Hanks' balanced salt solution (BSS) (*11*). Phosphate buffer (M/15) was added to give a final concentration of 25 mmoles per liter. Phenol red, $NaHCO_3$, penicillin, and streptomycin were not included in the medium. Initial *p*H was adjusted by the addition of HCl or NaOH. Substrates and inhibitors were included in the medium initially added to the reaction chamber of the Warburg vessel. Sodium lactate was prepared by neutralization of freshly distilled lactic acid with NaOH, with glass electrode and *p*H meter.

In other metabolic studies the tissues were incubated in a Ringer's bicarbonate medium (*12*) with glucose present in concentrations varying from 1.2 to 40 mmoles. Oxygen (95%) and CO_2 (5%) were employed as the gas phase for aerobic experiments, and nitrogen (95%) and CO_2 (5%) were used for anaerobic studies. Tissues were incubated at 37° C in stoppered Erlenmeyer flasks, and 10 minutes were allowed for equilibration of the liquid and gas phases. Samples for analysis were removed with a syringe and long needle by injection through the vaccine stopper. Approximately 800 mg of wet tissue was incubated in 15 ml of medium.

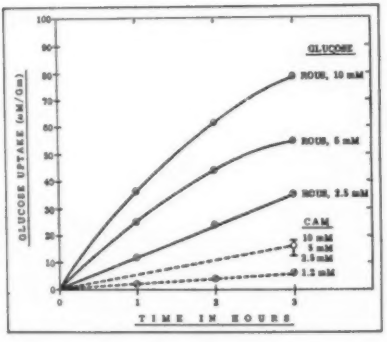
Initial and final concentrations of glucose were determined on deproteinized aliquots of medium by the Somogyi modification of the Nelson method (*13*). Concentrations of lactic acid were determined by the method of Barker and Summerson (*14*). The specific activity of the initial glucose was determined from the radioassay of the phenylosazone. Carbon dioxide was measured as $BaCO_3$ and isolated by acidification of the final medium and diffusion into alkali (*15*).

Glycogen content of the tissues was determined by digestion in KOH precipitation with ethanol, and hydrolysis of the glycogen with 5 N sulfuric acid (*16*). The glucose derived from glycogen was diluted with unlabeled glucose and isolated as the phenylosazone for radioassay. Tissue fatty acids were extracted from the ethanolic-KOH digest of the tissue after acidification with HCl (*17*). Fatty acids were plated on lens paper and counted.

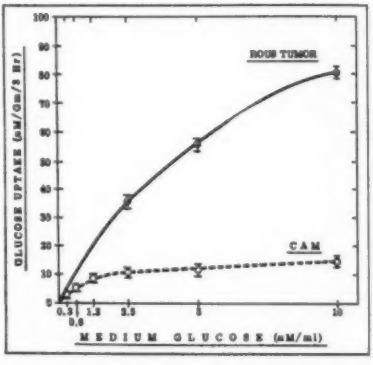
RESULTS

Rates of glucose utilization by RSV-tumor slices and control CAM were determined under aerobic and anaerobic conditions. Aliquots

of the incubation medium were removed at hourly intervals and analyzed for glucose. Essentially linear rates of utilization were observed for the various concentrations of glucose tested (text-fig. 1). The effect of substrate concentration on the rate of utilization is illustrated in text-figures 2 and 3. Glucose utilization by the tumor was considerably greater than that of the uninfected tissue at all concentrations tested. Under anaerobic conditions glucose utilization by tumor tissue decreased slightly, while utilization by the CAM markedly increased.



TEXT-FIGURE 1.—Glucose uptake by CAM and RSV tumor. Tissue slices were incubated in Ringer's bicarbonate medium with 95 percent oxygen and 5 percent CO_2 as the gas phase, and glucose was added to give final concentrations from 1.2 to 10 mmoles. Glucose uptake in μmoles per g of wet tissue has been plotted as a function of time. Each curve represents the mean of 2 or more experiments.



TEXT-FIGURE 2.—Aerobic glucose utilization by CAM and RSV tumor. Tissues were incubated in Ringer's bicarbonate medium with 95 percent oxygen and 5 percent CO_2 as the gas phase. Glucose uptake, expressed as μmoles of glucose utilized per g of tissue over a 3-hour incubation period, has been plotted as a function of the initial medium glucose concentration. Each point represents the mean of 2 or more experiments. Spread of values is given (Φ).

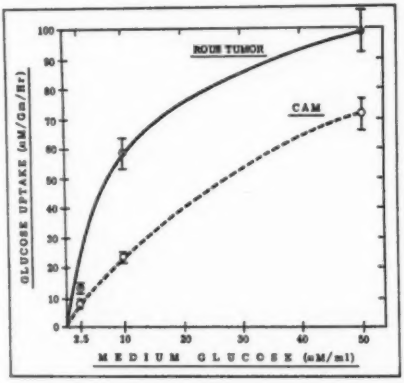
For further information regarding glucose utilization by these tissues, reciprocal plots, (1/V) versus (1/S), were made. From these curves, illustrated in text-figures 4*a* and *b*, apparent affinity constants and maximum rates of glucose utilization have been derived. In this system the RSV tumor has a maximum rate of glucose utilization 6 times that of the CAM. Half maximal velocity is achieved with 5 mmoles of glucose in the case of RSV tumor (text-fig. 4*a*), and with 1.3 mmoles of glucose by CAM (text-fig. 4*b*).

Carbon^{14}-glucose metabolism by the CAM is summarized in table 1. Under aerobic conditions 50 to 60 percent of the glucose utilized during the experimental period was recovered as CO_2. About 25 percent of the glucose could be considered lactic acid. Under anaerobic conditions lactic acid accounted for 30 to 80 percent of the glucose used, depending

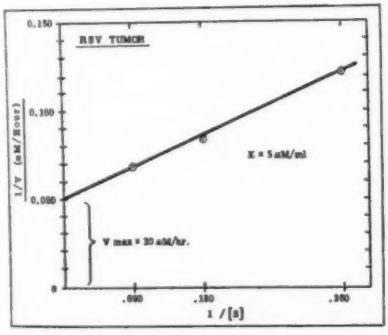
on the initial concentration of glucose present. With lower concentrations of glucose a greater percentage could be considered lactic acid.

In the RSV tumor only 5 to 6 percent of the glucose used was recovered as CO_2 (table 2). Lactate accounted for 50 to 100 percent of the glucose metabolized under both aerobic and anaerobic conditions. It was also observed that in the tumor, as well as the CAM, with lower medium concentrations of glucose more of the substrate was considered lactate.

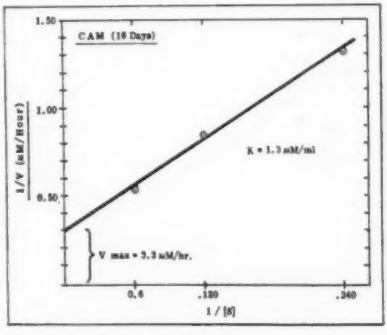
Glycogen and fat were determined, since these two tissue components represent the major energy stores. There was no significant difference



TEXT-FIGURE 3.—Anaerobic glucose utilization by CAM and RSV tumor. Conditions were identical with those in text-figure 2, except 95 percent nitrogen and 5 percent CO_2 were used for the gas phase. Glucose uptake over the 3-hour incubation period has been plotted as a function of the initial glucose concentration.



TEXT-FIGURE 4*a*.—Double reciprocal plot for rate of glucose utilization (1/V) and initial glucose concentration (1/S) in RSV tumor. Rates are expressed as μmoles of glucose utilized per hour per g of wet tissue, and glucose concentration is expressed as μmoles per ml.



TEXT-FIGURE 4*b*.—Double reciprocal plot for rate of glucose utilization (1/V) and initial glucose concentration (1/S) in CAM. Rates are expressed as μmoles of glucose utilized per hour per g of wet tissue and glucose concentration is expressed as μmoles per ml.

TABLE 1.—Metabolism of glucose-U-C^{14} by normal CAM (all values are expressed as μmoles per g wet tissue per 3-hour incubation)

Initial glucose concentration μmoles/ml	Number of observations	Glucose uptake	Lactic acid produced (average ± standard error)*	Glucose oxidized
Aerobic in 95 percent oxygen: 5 percent CO_2				
10	2	14.6	5.0	6.6
5	2	10.7	5.3	6.4
2.5	2	10.3	5.0	6.7
1.2	4	7.3 ±0.9	3.1 ±0.2	3.6 ±0.1
0.6	4	4.6 ±0.4	2.0 ±0.06	3.4 ±0.4
0.3	4	2.7 ±0.1	1.6 —	1.8 ±0.1
Anaerobic in 95 percent nitrogen: 5 percent CO_2				
50	6	71 ±5.5	46 ±6.4	
10	6	24 ±1.4	35 ±5.0	
2.5	6	7.4 ±0.6	12 ±4.7	

*Standard errors not computed for averages of only 2 observations.

TABLE 2.—Metabolism of glucose-U-C^{14} by RSV-tumor (all values μmoles per g wet tissue per 3-hour incubation)

Initial glucose concentration μmoles/ml	Number of observations	Glucose uptake	Lactic acid produced (average ± standard error)	Glucose oxidized
Aerobic				
10	6	80 ±2.4	96 ±5.6	4.4 ±0.8
5	6	55 ±0.9	74 ±3.2	2.7 ±0.5
2.5	4	35 ±1.6	41 ±2.7	2.2 ±0.4
Anaerobic				
50	6	99 ±7.6	83 ±20	
10	6	59 ±5.0	58 ±8.0	
2.5	6	12.5 ±0.6	30 ±3.4	

in the glycogen content of the two tissues (table 3). The amount of C^{14} from glucose deposited as glycogen represents only a small fraction of the glucose used by either tissue. Although the tumor tissue incorporated 10 to 20 times as much C^{14} into fatty acids as glycogen, only 5 percent of glucose utilized was recovered in fat. Little radioactivity was recovered in glycogen and fatty acids in the CAM.

In a consideration of the relationship of glucose to its suppressive effect on respiration of tumor tissue, data on the influence of the terminal

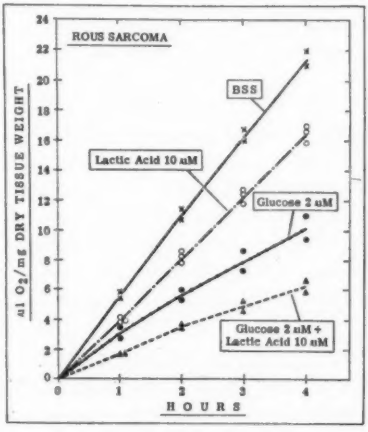
TABLE 3.—Incorporation of C^{14} from glucose into glycogen and fatty acids (all values μmoles per g wet tissue per 3-hour incubation)

Initial glucose concentration μmoles/ml	Number of observations	Glycogen		Glucose to fatty acids
		Total	From glucose	
		(average ± standard error)*		
RSV-tumor				
10	6	2.4 ±0.4	0.15 ±0.01	3.7 ±0.2
5	6	2.4 ±0.2	0.09 ±0.01	2.1 ±0.2
2.5	2	2.2	0.02	1.9
CAM				
10	2	2.4	0.04	0.09
5	2	2.1	0.05	0.07
2.5	2	2.5	0.04	0.08

*Standard errors not computed for averages of only 2 observations.

products of glycolysis, lactate, and hydrogen-ion concentration were of interest. Lactate suppressed oxygen uptake of RSV tumor when concentrations as low as 4 μmoles per ml were used. Addition of 10 μmoles of lactate per ml of medium did not yield the suppressive effect of 2 μmoles of glucose per ml on oxygen uptake. Quantitative conversion of all the glucose present to lactate would not account for the observed suppression of respiration. There was also an additive effect on suppression of oxygen uptake when glucose and lactate were together in the medium. These experiments are summarized in text-figure 5.

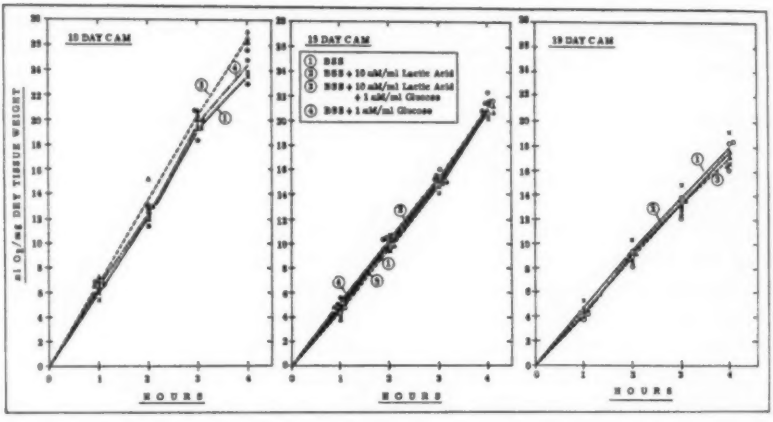
The oxygen uptake of CAM was not altered by 10 μmoles per ml of lactate alone or in the presence of 1 μmole of glucose per ml. Data of



TEXT-FIGURE 5.—Effect of lactate on RSV-tumor respiration in the presence and absence of glucose. Concentrations of substrates are given in μmoles per ml. Each point represents the average of triplicate results.

experiments comparing 10-, 15-, and 19-day-old CAM's are given in text-figure 6. Lactate or glucose did not significantly alter the rate of oxygen uptake observed as endogenous respiration when CAM was incubated in BSS.

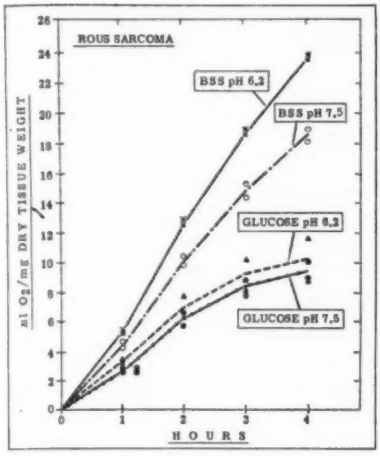
Initial adjustment of the medium to *p*H 6.2 did not enhance the suppressive effect of glucose on oxygen uptake of RSV tumor. However, the endogenous respiration of RSV tumor was increased (text-fig. 7). Adjustment of *p*H to 6.2 and 7.5 in experiments with and without 10 μmoles of glucose per ml gave final reactions of *p*H 5.5 with glucose and 7.6 for the endogenous system. The increased respiration of the endogenous system adjusted to an acid *p*H initially is in contrast to the usually observed suppression of O_2 consumption at lower *p*H.



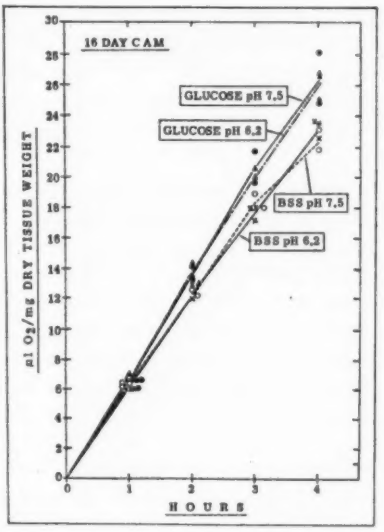
TEXT-FIGURE 6.—Effect of lactate on 10-, 15-, and 19-day-old CAM respiration in the presence and absence of glucose. Substrate concentration is given in μmoles per ml. Each point represents average of triplicate experiments.

Experiments with CAM did not show any effect of medium *p*H on respiration. Data given in text-figure 8 show that a concentration of 10 μmoles of glucose per ml gave a slight increase in oxygen uptake of 16-day CAM over the endogenous and that initial acid adjustment of the medium did not have an effect. The *p*H in the endogenous 16-day CAM changed from 6.2 to 7.6 and 7.5 to 7.8 during the incubation period. The final *p*H obtained in the presence of 10 μmoles of glucose per ml was *p*H 6.7 and 7.3, respectively, which suggests that glycolytic activity was not great.

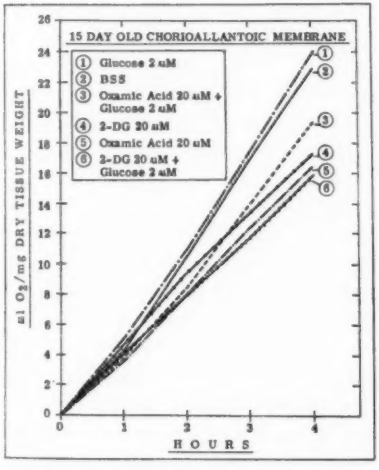
The suppressive effect of 2 deoxy-D-glucose (2DG) and oxamic acid on endogenous respiration of both RSV tumor and CAM, as shown in text-figures 9 and 10, emphasizes the significance of interference with glycolysis as a nonselective suppressive effect on respiration of tumor and nontumorous tissue. It is of interest that oxamic acid relieved the suppressive effect of glucose on tumor respiration. This result was not observed with



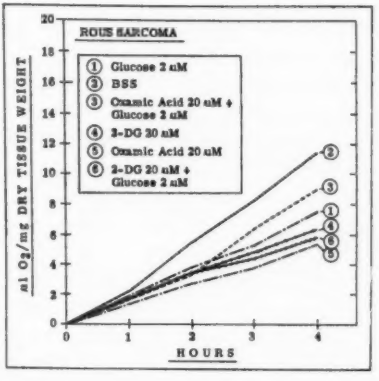
TEXT-FIGURE 7.—Effect of *p*H on respiration of RSV tumor in the presence of 10 µmoles of glucose per ml and in the absence of glucose. O_2 uptake determined after initial adjustment of *p*H as indicated. Each point represents average results of triplicate experiments.



TEXT-FIGURE 8.—Effect of *p*H on 16-day CAM in the presence of 10 µmoles of glucose per ml and in the absence of glucose as indicated. Each point represents average results of triplicate experiments.


15 DAY OLD CHORIOALLANTOIC MEMBRANE
1 Glucose 2 uM
2 BSS
3 Oxamic Acid 20 uM + Glucose 2 uM
4 2-DG 20 uM
5 Oxamic Acid 20 uM
6 2-DG 20 uM + Glucose 2 uM
µl O2/mg DRY TISSUE WEIGHT
HOURS

TEXT-FIGURE 9.—Effect of oxamic acid and 2DG on respiration of 15-day CAM in the presence and absence of glucose. Substrate concentrations are given in µmoles per ml. Each curve represents average results of triplicate experiments.


ROUS SARCOMA
1 Glucose 2 uM
2 BSS
3 Oxamic Acid 20 uM + Glucose 2 uM
4 3-DG 20 uM
5 Oxamic Acid 20 uM
6 2-DG 20 uM + Glucose 2 uM
µl O2/mg DRY TISSUE WEIGHT
HOURS

TEXT-FIGURE 10.—Effect of oxamic acid and 2DG on respiration of RSV tumor in the presence and absence of glucose. Substrate concentrations are given in µmoles per ml. Each curve represents average results of triplicate experiments.

2DG. Oxamic acid suppressed the endogenous respiration of both CAM and RSV tumor, as was observed with 2DG.

DISCUSSION

In this study involving quantitative estimates of rates of metabolism it has been observed that RSV infection of CAM produces pronounced alterations in glucose assimilation. Several points of difference should be particularly noted. In uninfected tissue CO_2 appears to be the major metabolic product of glucose metabolism, while in the RSV tumor (as well as other tumors of unknown etiology) lactate is the major metabolite. Kinetic studies indicate that RSV-tumor cells can utilize glucose at a rate 6 times that of CAM. Whether this increased rate of utilization is due to differences in rate of entrance of glucose into the cell or enzymatic changes has yet to be determined.

The present study described only two points (involving the metabolism of a single substrate) in the process of viral induction of neoplastic growth, *i.e.*, the uninfected cells and the fully developed tumor. This tumor of viral etiology exhibits metabolic features similar to other tumors. For example, oxygen consumption by tumor slices is depressed by the addition of glucose (Crabtree effect) and lactate production under aerobic conditions is increased. It was observed that glucose utilization is not increased under anaerobic conditions.

It is hoped that further studies in appropriate biological systems may yield more information relative to the sequence of events leading to the transformation of a cell to a neoplastic cell. In the present study the amount of glucose oxidized (μmoles/g wet tissue) was essentially the same in the tissue of origin and tumor tissue. In this respect the RSV tumor is similar to a a number of mammalian tumors examined by Wenner and Weinhouse (*18*). Because of the difference in rates of glucose utilization, the percentage of the glucose used that appears as CO_2 in the tumor slice is less than that observed with the chorio-allantois.

Since the potential for glucose oxidation remains relatively unchanged in the face of increased utilization, it might be argued that in this system lactate represents the potential of the glycolytic system relative to oxidative routes.

The possible role of lactate in the induction of a Crabtree effect was investigated 1) by determination of the respiration of tissues in the presence and absence of added lactate and 2) by the utilization of oxamic acid as an inhibitor of lactic dehydrogenase (*19*, *20*). Results shown in text-figures 5 and 6 indicate that lactate selectively suppresses oxygen uptake of RSV tumors, since the respiration of CAM was not altered by the presence of 10 μmoles per ml of lactate. The data show quantitatively that lactate is not as effective a suppressor of respiration of RSV tumor as glucose. The additive effect on suppression of respiration of the tumor (text-fig. 5) may represent a different mechanism of action. In the absence

of suppression by acidity (text-fig. 7) the role of lactate inhibition is of interest as its action was selective like that of glucose for RSV tumor, since it was not active on the respiration of CAM. The role of lactic dehydrogenase in the Crabtree effect is not apparent in that similar results were observed when the effect of oxamic acid on RSV tumors was compared with its effect on CAM (text-figs. 9 and 10).

The studies of Racker (*21*), Ibsen, Coe, and McKee (*22*), Quastel and Bickis (*23*), and Chance (*24*) suggest that increased glycolysis may inhibit oxygen consumption by reducing the intracellular concentrations of inorganic phosphate and adenosine diphosphate. This hypothesis is attractive when only tumor tissue is considered. Within certain limits an increase in glucose causes a further depression of oxygen consumption in RSV tumor (*6*). Glucose utilization *per se* does not seem to depress uptake of oxygen, since adjusting the initial glucose concentration yields comparable rates of utilization in the CAM and RSV tumor. Under these conditions only the tumor tissue exhibits a Crabtree effect.

Seelich and Letnansky (*25*) propose that the mechanism of the Crabtree effect is based on two presuppositions: 1) The substrates of the endogenous respiration are not carbohydrates but lipides. 2) The addition of glucose is followed not only by a change-over of the cell metabolism to this substrate but also by an increased participation of the shunt in the dehydrogenation of glucose when the glucose concentration is increasing. In the metabolism of glucose by the shunt, triphosphopyridine nucleotide (TPN) but not diphosphopyridine nucleotide (DPN) is reduced in contrast to glycolysis. Seelich and Letnansky postulate that the reduction of TPN is the determining factor in the inhibition of oxygen consumption by addition of glucose. "The dependence of the Crabtree effect on the glucose concentration and phosphate content of the medium occurs as a result of the turnover rate of glucose via the hexosemonophosphate pathway."

Unpublished studies from these laboratories comparing glucose-1-C^{14} and glucose-6-C^{14} metabolism by RSV tumor would indicate that an increase in glucose utilization does increase C^{14} incorporation into lipides. However, since C-1 and C-6 of glucose contribute equally to the lipide fraction, any extra glycolytic pathway would appear negligible. In addition, only 1 to 2 percent of the glucose utilized can be considered fat. Although C-1 of glucose is preferentially oxidized in this tumor, the shunt can account for no more than 4 percent of the glucose utilized.

REFERENCES

(*1*) Burk, D., Sprince, H., Spangler, J. M., Kabat, E. A., Furth, J., and Claude, A.: The metabolism of chicken tumors. J. Nat. Cancer Inst. 2: 201–240, 1941.

(*2*) Rous, P.: A transmissible avian neoplasm (sarcoma of the common fowl). J. Exper. Med. 12: 696–705, 1910.

(3) LEVINE, A. S., and EATON, M. D.: Decrease of respiration by glucose (Crabtree effect) in Rous sarcoma of chorioallantoic membrane. Proc. Soc. Exper. Biol. & Med. 100: 184–186, 1959.

(4) ASHMORE, J., UHL, R., and LEVINE, A. S.: Biochemical studies of virus-induced neoplastic growth. Science 130: 1411, 1959.

(5) WEBER, G., BANERJEE, G., LEVINE, A. S., and ASHMORE, J.: Metabolic studies on the Rous sarcoma. Proc. Am. Assoc. Cancer Res. (Abstract.) 3: 160, 1960.

(6) LEVINE, A. S., STRICKER, F., UHL, R., and ASHMORE, J.: Effect of glucose concentration on respiration of Rous sarcoma and of chorioallantoic membrane. Nature, London 188: 229–230, 1960.

(7) WAGLE, S. R., LEVINE, A. S., and ASHMORE, J.: Studies on the increased rate of protein synthesis in Rous sarcoma. 5th Internat. Biochem. Cong. Moscow, 1961. In press.

(8) CRABTREE, H. G.: Observations on the carbohydrate metabolism of tumours. Biochem. J. 23: 536–545, 1929.

(9) KEOGH, E. V.: Ectodermal lesions produced by the virus of Rous sarcoma. Brit. J. Exper. Path. 19: 1–9, 1938.

(10) PRINCE, A. M.: Quantitative studies on Rous sarcoma virus. II. Mechanism of resistance of chick embryos to chorio-allantoic inoculation of Rous sarcoma virus. J. Nat. Cancer Inst. 20: 843–850, 1958.

(11) HANKS, J. H., and WALLACE, R. E.: Relation of oxygen and temperature in the preservation of tissues by refrigeration. Proc. Soc. Exper. Biol. & Med. 71: 196–200, 1949.

(12) CAHILL, G. F., Jr., ASHMORE, J., ZOTTU, S., and HASTINGS, A. B.: Studies on carbohydrate metabolism in rat liver slices. IX. Ionic and hormonal effects on phosphorylase and glycogen. J. Biol. Chem. 224: 237–250, 1957.

(13) SOMOGYI, M.: Determination of blood sugar. J. Biol. Chem. 160: 69–73, 1945.

(14) BARKER, S. B., and SUMMERSON, W. H.: The colorimetric determination of lactic acid in biological material. J. Biol. Chem. 138: 535–554, 1941.

(15) ASHMORE, J., KINOSHITA, J. H., NESBETT, F. B., and HASTING, A. B.: Studies on carbohydrate metabolism in rat liver slices. VII. Evaluation of the Embden-Meyerhof and phosphogluconate oxidation pathways. J. Biol. Chem. 220: 619–626, 1956.

(16) GOOD, C. A., KRAMER, H., and SOMOGYI, M.: The determination of glycogen. J. Biol. Chem. 100: 485–491, 1933.

(17) RENOLD, A. E., HASTINGS, A. B., NESBETT, F. B., and ASHMORE, J.: Studies on carbohydrate metabolism in rat liver slices. IV. Biochemical sequence of events after insulin administration. J. Biol. Chem. 213: 135–146, 1955.

(18) WENNER, C. E., and WEINHOUSE, S.: Metabolism of neoplastic tissue. IX. An isotope tracer study of glucose catabolism pathways in normal and neoplastic tissues. J. Biol. Chem. 222: 399–414, 1956.

(19) NOVA, W. B., WINER, A. D., GLAID, A. J., and SCHWERT, G. W.: Lactic dehydrogenase. V. Inhibition by oxamate and by oxalate. J. Biol. Chem. 234: 1143–1148, 1959.

(20) PAPACONSTANTINOU, J., and COLOWICK, S. P.: Effects of oxamic acid on metabolism of ascites tumors. Fed. Proc. 16: 230–231, 1957.

(21) RACKER, E.: Carbohydrate metabolism in ascites tumor cells. Ann. New York Acad. Sc. 63: 1017–1021, 1956.

(22) IBSEN, K. H., COE, E. L., and McKEE, R. W.: Interrelationships of metabolic pathways in Ehrlich ascites carcinoma cells. I. Glycolysis and respiration (Crabtree effect). Biochem. et biophys. acta. 30: 384–400, 1958.

(23) QUASTEL, J. H., and BICKIS, I. J.: Metabolism of normal tissues and neoplasms *in vitro*. Nature, London 183: 281–286, 1959.

(24) CHANCE, B.: Regulation of cell metabolism. Ciba Foundation Symp. (Wolstenholme, G. E. W., and O'Connor, C. M., eds.). Boston, Little, Brown & Co., 1959, pp. 91–129.

(25) SEELICH, F., and LETNANSKY, K.: Über die Abhängigkeit des Sauerstoff Verbrauches von Ehrlich-Ascites-Carcinomzellen von der Oxydation der Glucose über den Hexose-Monophosphate Shunt. Untersuchungen zur Frage des Reaktionsmechanismus des "Crabtree-Effektes." Ztschr. Krebsforsch. 63: 236–248, 1960.

Carcinogenesis of the Uterine Cervix in Mice: Effect of Diethylstilbestrol After Limited Application of 3-Methylcholanthrene [1, 2]

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SUMMARY

Interrelationships between a chemical carcinogen and hormonal status in cervical carcinogenesis were investigated. No significant effect of castration or added estrogen stimulation was detected under conditions of prolonged exposure to methylcholanthrene by cervical painting or continuous exposure of the endocervical epithelium to a methylcholanthrene-coated thread. When the time of exposure to methylcholanthrene-coated threads was reduced to 4 weeks, a low incidence of cervical carcinoma appeared in castrates, providing a base line for determination of the effect of subsequent systemic treatment with estrogenic hormone. The incidence of cervical carcinoma was increased and the average latent period decreased in strains A/Sn, C3HeB/FeJ, and 129/J when systemic treatment with diethylstilbestrol followed a 4-week exposure of the castrate endocervical epithelium to a methylcholanthrene-coated thread. The incidence of carcinoma increased from 13.2 to 90.2 percent when 33 percent diethylstilbestrol-cholesterol pellets were administered. The average latent period was reduced from 12.3 to 6.7 months. Ten percent diethylstilbestrol-cholesterol pellets increased the incidence of carcinoma from 3.3 to 19.1 percent with lesser shortening of the average latent period. Plain-thread stimulation of the castrate endocervical epithelium for 4 weeks followed by 33 percent diethylstilbestrol pellets produced an incidence of 7.1 percent carcinoma in the three strains. Ten percent diethylstilbestrol pellets produced carcinoma in 1.5 percent of strain A castrates. Plain-thread stimulation for 4 weeks produced no carcinomas among 78 mice. The "promoting" action of estrogen in these experiments is discussed.—J. Nat. Cancer Inst. 27: 611–653, 1961.

THE EXPERIMENTAL induction of carcinoma of the uterine cervix has been approached in two directions: direct application of carcinogenic agents and systemic administration of estrogenic hormones.

Direct application of crude-tar preparations to the cervix and vagina in mice and rats produced only a few carcinomas (*1–3*). After isolation of the more potent carcinogenic hydrocarbons, attempts at intravaginal application of these substances in oily solutions were either reported as

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[2] Supported in part by grant E-34 from the American Cancer Society, Inc., and a grant from the Elsa U. Pardee Foundation. Initial studies were carried out in the Laboratory of Pathology, National Cancer Institute, National Institutes of Health, Bethesda, Md.

negative (*3*, *4*) or produced many more tumors of the vulva and perineum than of the vagina (*5*).

The demonstration of induction of carcinoma of the vagina and cervix in mice surviving prolonged systemic treatment with estrogenic hormones (*6*, *7*) provided the dominant theme in the field of experimental cervical carcinogenesis for over a decade. Gardner reported a number of studies of this phenomenon (*8*, *9*) and recently showed that direct intravaginal application of estrogen is more effective that systemic administration in the induction of carcinoma (*10*).

In the first study accomplishing a high yield of squamous-cell carcinoma of the vagina, benzo[*a*]pyrene dissolved in cholesterol was used (*11*). Fragments of this preparation were repeatedly inserted into the vaginas of strain A mice. Investigators have attempted more concentrated exposure of the cervix to chemical carcinogens by: transplanting cervical tissues in contact with methylcholanthrene (*12*), painting the visualized cervix with methylcholanthrene dissolved in acetone (*13*), applying benzo[*a*]pyrene in acetone solution to the cervix with a wire-loop applicator (*14*, *15*), inserting carcinogen-coated threads into the endocervical canal (*13*, *16*, *17*), and inserting carcinogen-containing pellets in the vagina (*10*).

The endocervical-thread technique has given the highest yields of cervical carcinoma and the shortest latent periods (*13*, *16*, *18*, *19*). The development of an efficient method for induction of cervical carcinoma by means of chemical carcinogens permits an approach to the problem of the possible interrelationships of chemical carcinogens and hormones in cervical carcinogenesis (*18*, *19*). This study analyzes the effects of limited application of methylcholanthrene-coated threads followed by systemic administration of estrogenic hormone, in comparison with the effects of prolonged exposure to methylcholanthrene.

MATERIALS AND METHODS

Three inbred strains of mice, A/Sn, C3HeB/FeJ, and 129/J, were used in the major experiments involving limited exposure to methylcholanthrene-coated and uncoated threads placed in the endocervical canal. Females were castrated at 1.5 to 4 months of age and received threads 1 month later. The exploratory experiments involving methylcholanthrene painting, continuous exposure to methylcholanthrene-coated threads, and limited exposure to methylcholanthrene-coated threads were done with mice of sublines A/He, C3Hf/He, and C57BL/He, 3 to 5 months of age.

Cervices were painted with a 0.3 percent solution of 3-methylcholanthrene dissolved in acetone. A fine cotton swab on a toothpick was moistened lightly with the solution and applied twice weekly to the cervix, visualized by means of an otoscope equipped with an infant-sized speculum (fig. 1). Painting was continued for 10 months. Controls were painted in similar fashion with acetone alone, through a speculum that never came in contact with the carcinogen.

Size 5–0 black silk surgical threads were dipped into melted methylcholanthrene, which produced a thin coating of the carcinogen on approximately 3 mm of the end of the thread for continuous exposure. If threads were to be removed subsequently through the vagina, the coated segment was placed approximately 5 mm from the tip as a small loop of the thread was dipped into the melted methylcholanthrene. The threads were autoclaved and inserted into the endocervical canal by means of a combined vaginal and abdominal operation, with anesthetized animals immobilized on a vertically adjustable platform. A blunted 1.5 cm length of a Sharps size-10 sewing needle bearing the thread was mounted in a 19-gauge trocar. The needle was introduced through the vagina into the external os of the endocervical canal, visualized by means of the otoscope. The tip of the needle was guided through the endocervical canal past the squamocolumnar junction at the internal os into the right uterine horn by observation through a lower abdominal incision. At a point just above the external bifurcation of the uterus, the tip of the needle was forced medially through the uterine wall. We drew the thread through until we saw the coated portion pass just within the external os of the cervix. Permanent threads were secured by incorporation into a knot of white cotton thread. Threads designed to be removed through the vagina were held in place by the friction of a triple knot of cotton thread tied around the silk thread where it emerged from the uterine wall. Thus the exposure to methylcholanthrene tended to be limited to the stratified squamous epithelium of the endocervical canal, the squamocolumnar junction, and a short segment of the glandular mucosa of the uterus (fig. 2). The upper vagina and the myometrium usually came in contact only with the uncoated portions of the thread. Animals in which threads were lost were eliminated from the experiments.

Diethylstilbestrol-cholesterol pellets were prepared by the method of Shimkin and White (*20*). Segments weighing approximately 5 mg were cut from cylinders composed of 33 and 10 percent diethylstilbestrol dissolved in melted cholesterol. The pellets were introduced subcutaneously in the interscapular region by means of a trocar at the time of thread removal. In the continuous endocervical-thread experiments the pellets were introduced at the time of thread insertion. Animals in which the pellets were not demonstrated at autopsy were eliminated from the experiments.

At autopsy, the reproductive tract, lumbar and caudal lymph nodes, left lung, kidneys, liver, spleen, and the adrenal, pituitary, and submaxillary glands were routinely fixed in either Zener-formol fluid or Fekete's acetic alcohol formalin fluid. In addition, enlarged intra-abdominal lymph nodes, grossly pathological organs, and extragenital tumors were prepared for microscopic examination. The reproductive tract was fixed *in toto* and cut into multiple blocks after hardening. For the painting experiments, full serial sections of the entire cervix and upper vagina were obtained on nontumorous-appearing reproductive tracts. The lower half of the vagina and the vulva were sectioned longitudinally. Larger tumors

appearing in these experiments were studied by multiple blocks that included adjacent structures. In the endocervical-thread experiments, the region from the upper vagina to just above the external bifurcation of the uterus was divided transversely into slices approximately 1.5 mm in thickness, in the absence of obvious tumor. The vagina and vulva were sectioned longitudinally. Blocks were embedded in paraffin, sectioned, and stained routinely with hematoxylin and eosin. Selected sections of tumors were stained by the periodic acid-Schiff method, Mallory's phosphotungstic acid-hematoxylin method, and Wilder's reticulum method, and were examined under polarized light.

For the sake of uniform statistical comparison of the cervical tumors induced in these experiments, an arbitrary distinction is made between squamous-cell carcinomas larger than 1.5 mm in average dimension, as measured in histological sections, and smaller invasive lesions. The larger lesions are all interpreted as unquestionably malignant by histological and cytological criteria. The average cervical carcinoma in these experiments was much larger than 1.5 mm, even after the shrinkage of paraffin embedding. Superficial preneoplastic and neoplastic changes, and invasive-appearing lesions measuring less than 0.3 mm in average diameter, were eliminated from the final tabulation of the data. The method of sampling by means of 1.5 mm blocks could have missed smaller lesions at a rate in inverse proportion to their size. Most of the microscopic invasive lesions are interpreted as unquestionably malignant in terms of histological and cytological criteria. However, a few lesions occurring during the course of estrogen stimulation, while appearing invasive, were cytologically quite uniform. The arbitrary dividing line of size could have been drawn at a series of other points in the analysis of these experiments, including the four stages used in the analysis of estrogen-induced lesions, without changing the conclusions of the study.

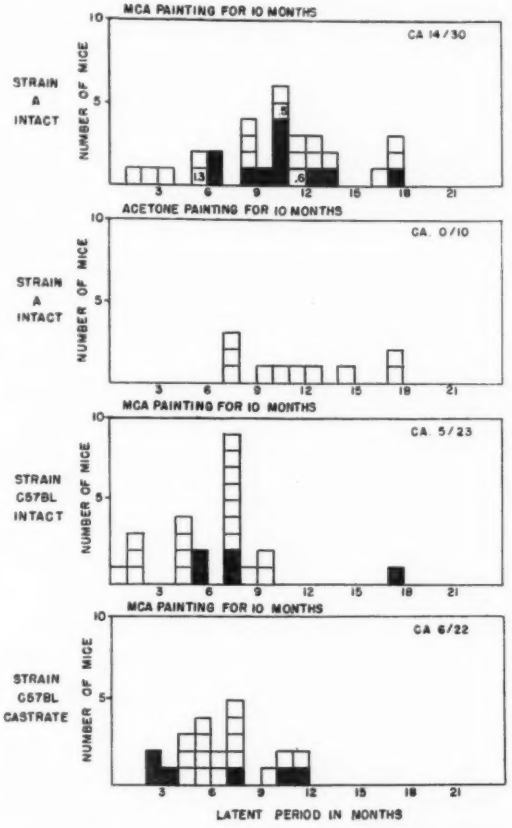
Animals were checked daily, and for the sake of good histological material, were killed when they had a palpable abdominal mass or appeared terminally ill.

RESULTS

Prolonged Application of Methylcholanthrene

Painting experiments.—Invasive squamous-cell carcinomas of the cervix and vagina were induced in 14 of 30 strain A/He intact virgin females that were painted twice weekly with a solution of methylcholanthrene in acetone (text-fig. 1, table 1). Eleven tumors ranged from 2 to 10 mm in average dimension in histological sections. The larger tumors involved the cervix and upper vagina. The smaller tumors could be localized on the cervix, in the fornices, and in the upper- and mid-vagina. Serial sections revealed additional atypical epithelial changes and focal invasive lesions of the cervix and vagina which were excluded from the final tabulation. Among the larger tumors, invasion of the rectum occurred in six instances

and invasion of the bladder in one. Vascular invasion was demonstrated in 9 tumors; however, pulmonary metastasis was found in only 1 animal. No metastasis was noted in the regional lymph nodes. There were 2 papillary tumors of the vulva. A pelvic abscess occurred in 1 animal and a rectovaginal fistula in another.



TEXT-FIGURE 1.—CERVICAL PAINTING. Incidence and latent period of squamous-cell carcinoma of the cervix and vagina and survival, in intact and castrate mice, painted twice weekly for 10 months with 0.3 percent methylcholanthrene in acetone or with acetone alone. In all the text-figures, *black squares* represent carcinomas >1.5 mm in mean dimension; *squares containing numbers* indicate the mean dimension in mm of smaller invasive lesions; *white squares* represent animals with no carcinoma demonstrated histologically.

In histological type, the squamous-cell carcinomas ranged from a majority that were well differentiated, showing extensive keratinization, to a few that were poorly differentiated and showed little or no keratinization. The average latent period of the carcinomas of the cervix and vagina was 10.4 ± 0.80 months (table 1).

TABLE 1.—Cervical painting: incidence and latent period of squamous-cell carcinoma of cervix and vagina and survival of mice painted with 3-methylcholanthrene and acetone

Procedure	Gonadal status	Strain	Number of mice	Squamous-cell carcinoma >1.5 mm	Squamous-cell carcinoma <1.5 mm*	Percent carcinoma	Average latent period carcinoma (months ± SE)†	Average survival (months ± SE)†
MCA painting‡ for 10 months	Intact	A	30	11	3	47	10. 4 ± 0. 80	10. 3 ± 0. 76
Acetone painting for 10 months	Intact	A	10	0	0			11. 7 ± 1. 20
MCA painting‡ for 10 months	Intact	C57BL	23	5	0	22	8. 7 ± 2. 40	6. 8 ± 0. 70
MCA painting‡ for 10 months	Castrate	C57BL	22	6	0	27	6. 6 ± 1. 73	6. 9 ± 0. 59

*Average dimension of tumors as measured in sections.

†Standard error of the mean.

‡Painted twice weekly with 0.3 percent methylcholanthrene dissolved in acetone.

Strain C57BL/He mice tolerated the painting procedure poorly. Pelvic abscesses occurred in 9 of 23 intact females and 11 of 22 castrates, and rectovaginal fistulas in 2 of the intact animals and 10 of the castrates. The average survival was shortened by these secondary complications. Invasive squamous-cell carcinomas of the cervix and vagina occurred in 5 of 23 intact females at an average latent period of 8.7 ± 2.40 months and in 6 of 22 castrates at an average latent period of 6.6 ± 1.73 months (table 1).

The squamous-cell carcinomas in strain C57BL resembled those in strain A in range of differentiation, with the exception that foci of mucin-containing cells replaced pearls of keratin in 3 tumors in the castrates. Pulmonary metastasis was observed in the case of the largest tumor appearing among the castrates, at a latent period of 80 days. Four castrates developed sarcomas involving the cervix and vagina at an average latent period of 7.3 months.

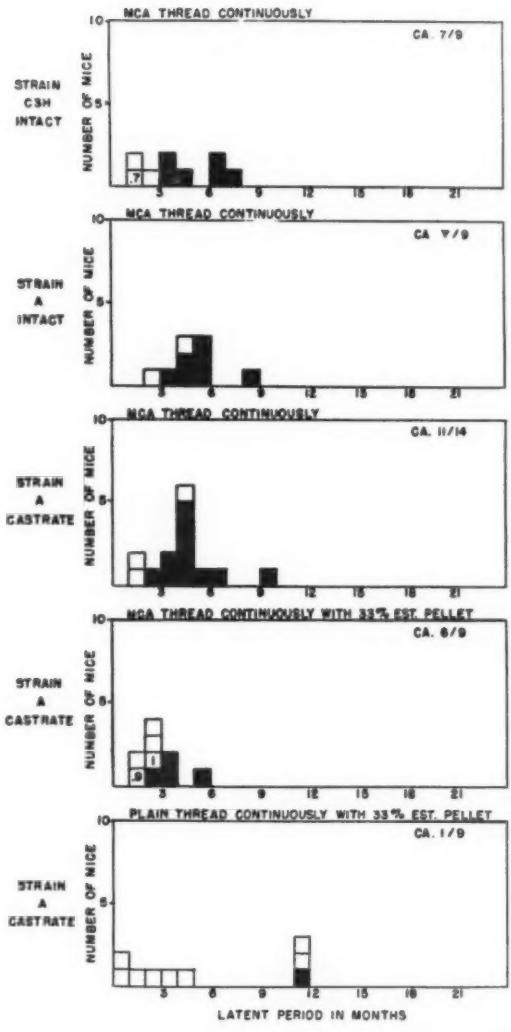
Ten strain A intact females were painted twice weekly with acetone alone for 10 months. One rectovaginal fistula occurred and one instance of ulceration of the cervix. There were no carcinomas or cytologically atypical proliferations.

While painting of the visualized cervix with continued light applications of carcinogen induced a number of carcinomas primarily involving the cervix, there were also a number of carcinomas primary in the vagina. The yield was under 50 percent and the average latent periods were long. It appeared that the localization of the exposure to carcinogen might be made more precise, the stimulus intensified, and the human disease more closely simulated anatomically by restricting the exposure to the endocervical canal.

Continuous endocervical thread.—In comparison with the painting procedure, the incidence of squamous-cell carcinoma of the cervix was increased and the average latent period reduced by insertion into the endocervical canal of a silk thread impregnated with melted methylcholanthrene (text-figure 2, table 2).

In intact virgin females, 7 carcinomas appeared among 9 strain C3Hf/He animals at an average latent period of 4.9 ± 0.81 months, and 7 carcinomas among 9 strain A animals at an average latent period of 5.3 ± 0.66 months. Strain A castrates developed 11 cervical carcinomas among 14 mice at an average latent period of 4.9 ± 0.57 months. When strain A castrates received in addition a subcutaneous pellet containing 33 percent diethylstilbestrol, 6 of 9 animals developed cervical carcinoma at an average latent period of 3.4 ± 0.55 months. A sarcoma of the cervix occurred in one of the strain A castrates in association with a carcinoma of the cervix.

The squamous-cell carcinomas presented a broad range of differentiation, but usually showed some degree of keratin formation. The tumors originated within the endocervical canal (figs. 3 and 4), as clearly demonstrated by serial block sections of the cervical region (figs. 5 and 6).



TEXT-FIGURE 2.—CONTINUOUS ENDOCERVICAL THREAD. Incidence and latent period of squamous-cell carcinoma of the cervix and survival in intact and castrate mice receiving endocervical threads either thinly coated with methylcholanthrene or uncoated. In addition, two groups received 33 percent diethylstilbestrol pellets subcutaneously.

The squamous-cell carcinomas of the cervix induced by carcinogen-coated threads are highly invasive. The larger tumors commonly invade the upper vagina, uterine horns, and paracervical fatty tissue. Rectal (fig. 7) and bladder invasion (fig. 8) occur; however, rectal invasion is less common than with tumors induced by cervical painting, in which extensive

TABLE 2.—Continuous endocervical thread: incidence and latent period of squamous-cell carcinoma of cervix and survival

Procedure	Gonadal status	Strain	Number of mice	Squamous-cell carcinoma >1.5 mm	Squamous-cell carcinoma <1.5 mm*	Percent carcinoma	Average latent period carcinoma (months ± SE) †	Average survival (months ± SE) †
MCA thread continuously	Intact	C3H	9	6	1	78	4.9 ± 0.81	4.2 ± 0.76
MCA thread continuously	Intact	A	9	7	0	78	5.3 ± 0.66	5.0 ± 0.57
MCA thread continuously	Castrate	A	14	11	0	78	4.9 ± 0.57	4.3 ± 0.56
MCA thread continuously with 33 percent diethylstilbestrol pellet‡.	Castrate	A	9	4	2	67	3.4 ± 0.55	3.0 ± 0.44
Plain thread continuously with 33 percent diethylstilbestrol pellet‡	Castrate	A	9	1	0	11	11.6	4.5 ± 1.63

*Average dimension of tumors as measured in sections.

†Standard error of the mean.

‡Diethylstilbestrol-cholesterol pellets weighed 5 mg and were inserted subcutaneously.

vaginal involvement is more common. Invasion of blood vessels (fig. 9) was frequently observed at the periphery of many of the larger tumors, but metastatic growths (fig. 10) were rarely seen in random sections of the lung. Metastasis to regional lymph nodes is rare. Direct invasion of contiguous nodes is more common. The tumors are transplantable (fig. 11) within the inbred strain of origin.

As a control for the group of strain A castrates receiving both methylcholanthrene-coated threads and 33 percent diethylstilbestrol-cholesterol pellets, 9 strain A castrates received plain silk endocervical threads and 33 percent diethylstilbestrol pellets. No tumors were found among the animals dying within the latent period of carcinoma of the preceding group (text-fig. 2). One cervical carcinoma appeared among 3 mice that survived 11 months of treatment (figs. 12 and 13). Cytologically, it was well differentiated.

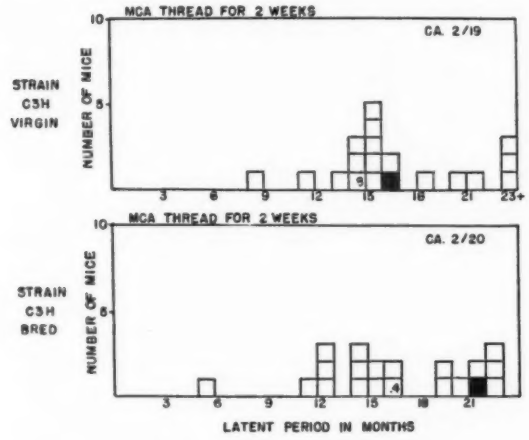
Continuous exposure to a carcinogen-coated endocervical thread produced primary squamous-cell carcinoma of the cervix in 78 percent of the animals, with average latent periods of approximately 5 months.

Limited Application of Methylcholanthrene

The endocervical-thread technique produced the highest yields of cervical carcinoma with the shortest latent periods and offered the possibility of shortening the time of exposure to the carcinogen in the exploration of possible cocarcinogenic and promoting factors that might be involved in the process of carcinogenesis in this hormone-responsive epithelium. The apparent lower sensitivity of the cervical and vaginal epithelium as compared with skin suggested that single cervical painting would not likely produce an adequate incidence of tumors for such studies.

Endocervical thread for 2 weeks.—Strain C3Hf virgin females, 2 to 3 months of age, received a methylcholanthrene-coated thread that was removed at the end of a 2-week period, after which half of the animals were continually caged with males. Litters were removed shortly after birth. The results are summarized in text-figure 3 and table 3. One carcinoma of the cervix, measuring 3 mm in average dimension, was found among 19 virgin females 16.6 months after the beginning of the exposure to the thread. In addition, a microscopic carcinoma measuring 0.8 mm in diameter was observed at 14.7 months. Among 20 bred females, a cervical carcinoma measuring 10 mm in average dimension was noted at 21.3 months. In addition, a microscopic carcinoma measuring 0.4 mm in diameter was observed at 16.3 months. Bred females had an average of 4.1 litters (1 to 7). The tumors occurred in animals that had 3 litters each. Pyometra was observed in 1 bred female at 16.2 months.

Under these conditions in the mouse there was no evidence that single or multiple pregnancy enhanced the development of carcinoma in a cervix exposed to methylcholanthrene for 2 weeks. However, within the short lifespan of the mouse the incidence of cervical carcinoma was too low to



TEXT-FIGURE 3.—MCA THREAD FOR 2 WEEKS. Incidence and latent period of squamous-cell carcinoma of the cervix and survival in virgin and breeding mice that had received a methylcholanthrene-coated thread left in the endocervical canal for 2 weeks.

analyze in a 2-week exposure of the cyclic endocervical epithelium to a methylcholanthrene-coated thread. A more intense initiating stimulus should be investigated.

No tumors occurred within the lifespan of 20 strain A castrates exposed to methylcholanthrene-coated thread for 2 weeks (table 3).

Endocervical thread for 4 weeks.—No carcinomas occurred among 12 strain C3Hf intact females observed as long as 14 months (text-fig. 4, table 3). One sarcoma of the cervix was found in the longest survivor. Unfortunately, the curve was truncated by the elimination of 5 additional animals from the experiment at this critical time by a weekend water-bottle accident.

Three cervical carcinomas occurred among 18 strain C3Hf castrates at widely scattered latent periods. The earliest tumor measured 1.4 mm in average diameter at a latent period of 28 days (text-fig. 4). It apparently arose in a sinus tract above the cervix and along the thread, preventing the ready removal of the thread. In addition, a sarcoma of the cervix and a sarcoma and an adenocarcinoma of the lower segment of one uterine horn were observed among these animals. No pelvic abscesses occurred in this group of mice.

Twelve C3Hf castrates received a 33 percent diethylstilbestrol-cholesterol pellet subcutaneously at the time of removal of the methylcholanthrene-coated endocervical thread. Seven carcinomas were found at an average latent period of 4.2 $\pm$ 0.33 months, an incidence of 58 percent (text-fig. 4, table 3). The incidence and latent period of cervical carcinoma were similar to the findings with continuous methylcholanthrene-coated threads. A representative tumor is illustrated (figs. 14 and 15). Pyometra and pelvic abscesses were common in this group. In addition to

TABLE 3.—Limited endocervical thread exposure: incidence and latent period of squamous-cell carcinoma of cervix and survival

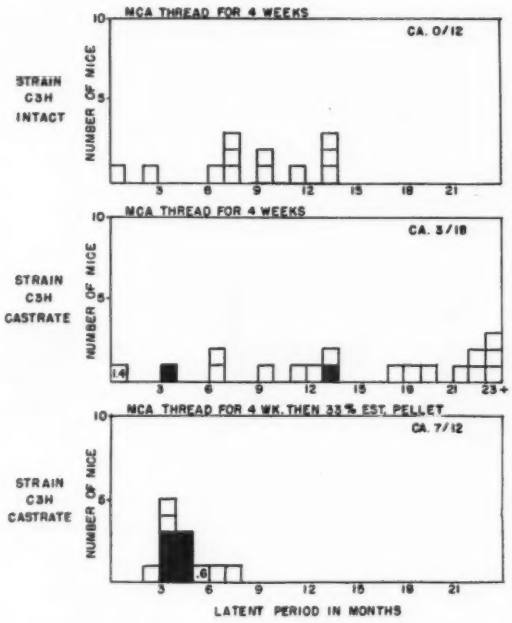
Procedure	Gonadal status	Strain	Number of mice	Squamous-cell carcinoma >1.5 mm	Squamous-cell carcinoma <1.5 mm*	Percent carcinoma	Average latent period carcinoma (months ± SE)†	Average survival (months ± SE)†
MCA thread for 2 weeks	Intact (virgin)	C3H	19	1	1	11	15.6 ± 0.97	17.1 ± 1.08
MCA thread for 2 weeks	Intact (bred)‡	C3H	20	1	1	10	18.8 ± 2.50	16.6 ± 1.04
MCA thread for 2 weeks	Castrate	A	20	0	0			13.8 ± 0.97
MCA thread for 4 weeks	Intact	C3H	12	0	0			8.8 ± 1.19
MCA thread for 4 weeks	Castrate	C3H	18	2	1	17	5.8 ± 3.78	15.6 ± 1.82
MCA thread for 4 weeks, then 33 percent diethylstilbestrol pellet §	Castrate	C3H	12	6	1	58	4.2 ± 0.33	4.5 ± 0.46

* Average dimension of tumors as measured in sections.

† Standard error of the mean.

‡ Bred females had from 1 to 7 litters, averaging 4.1.

§ Diethylstilbestrol-cholesterol pellets weighed 5 mg and were inserted subcutaneously.



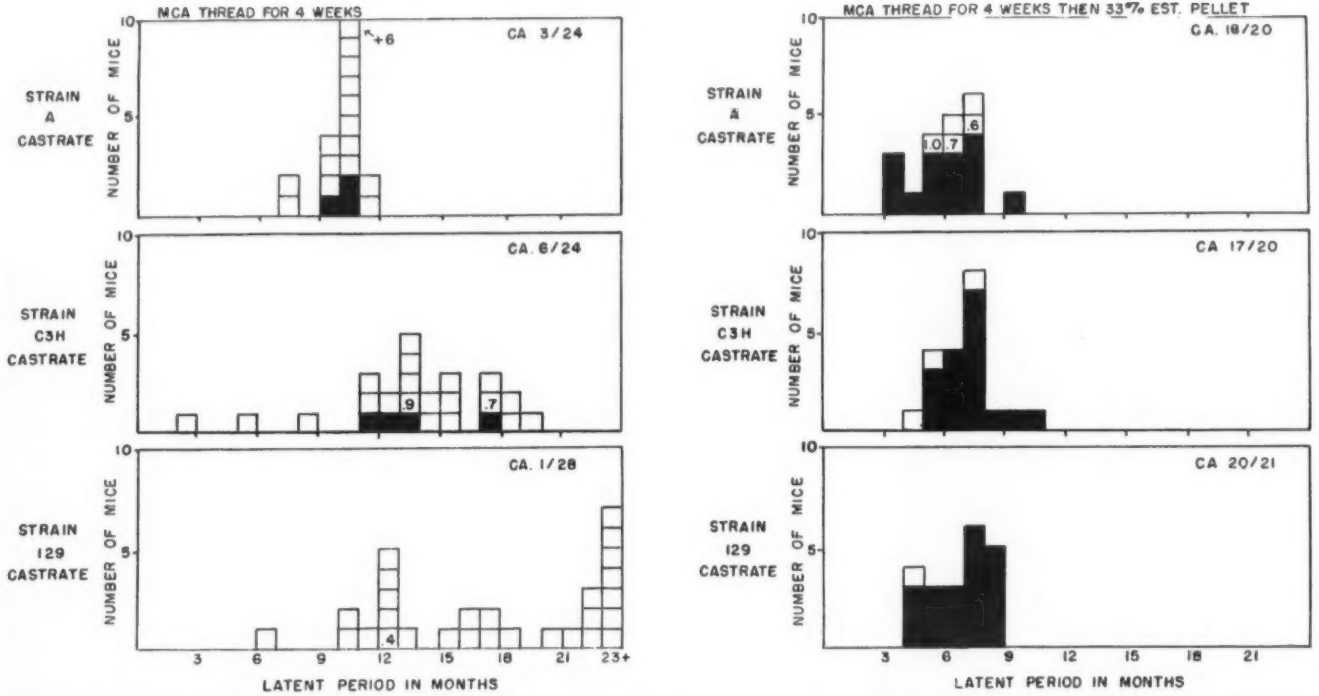
TEXT-FIGURE 4.—MCA THREAD FOR 4 WEEKS. Incidence and latent period of squamous-cell carcinoma of the cervix and survival in intact and castrate mice that had received a methylcholanthrene-coated thread left in the endocervical canal for 4 weeks. One group of castrates received in addition a 33 percent diethylstilbestrol pellet subcutaneously at the time of thread removal.

the cervical carcinomas, 2 cervical sarcomas were observed. While the toxic effects of this large dose of estrogen greatly shortened the lifespan of these castrates, the estrogen at the same time markedly increased the incidence of cervical carcinoma within the available latent period.

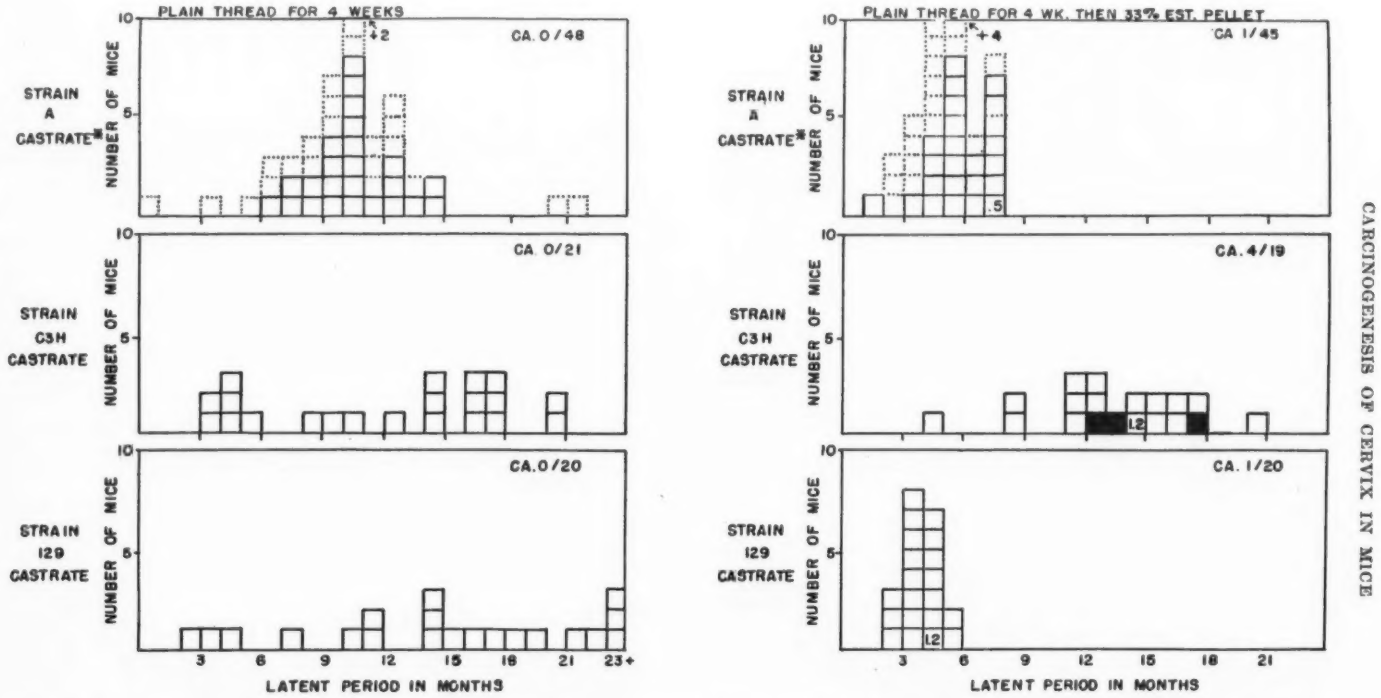
Thread in Castrates 4 Weeks, Then 33 Percent Estrogen Pellet

The results of the preceding experiment have been confirmed for the three inbred strains of mice tested, A/Sn, C3HeB/FeJ, and 129/J, and plain-thread controls were evaluated (text-figs. 5 and 6, table 4). Females were castrated at 2 to 4 months of age and, after a rest period of 4 weeks, received endocervical threads for 4 weeks. At the time of removal of the threads, the odd-numbered animals received 33 percent diethylstilbestrol-cholesterol pellets subcutaneously.

Methylcholanthrene-coated thread alone.—The base-line incidence of cervical carcinoma in castrates exposed to methylcholanthrene-coated threads for 4 weeks, but receiving no estrogen treatment, ranged from 4 percent for strain 129 to 25 percent for strain C3HeB (table 4). Three carcinomas occurred among 24 strain A castrates at an average latent period of 10.1 $\pm$ 0.49 months. Six carcinomas occurred among 24 strain



TEXT-FIGURE 5.—MCA THREAD FOR 4 WEEKS IN CASTRATES. Incidence and latent period of squamous-cell carcinoma of the cervix and survival in castrates that had received a methylcholanthrene-coated thread left in the endocervical canal for 4 weeks. Alternate animals in addition received a 33 percent diethylstilbestrol pellet subcutaneously at the time of thread removal.



TEXT-FIGURE 6.—PLAIN THREAD FOR 4 WEEKS IN CASTRATES. Incidence and latent period of squamous-cell carcinoma of the cervix and vagina and survival in castrates that had received a plain thread left in the endocervical canal for 4 weeks. Alternate animals in addition received a 33 percent diethylstilbestrol pellet subcutaneously at time of thread removal. * *White squares with dotted lines* indicate mice subjected to supracervical hysterectomy at time of castration. No carcinomas were demonstrated histologically in the latter groups.

TABLE 4.—Methylcholanthrene-coated and plain endocervical threads for 4 weeks in castrates with and without subsequent *33 percent diethylstilbestrol pellets:* incidence and latent period of squamous-cell carcinoma and survival

Procedure	Gonadal status	Strain	Number of mice	Squamous-cell carcinoma >1.5 mm	Squamous-cell carcinoma <1.5 mm*	Percent carcinoma	Average latent period carcinoma (months ± SE)†	Average survival (months ± SE)†
MCA thread for 4 weeks	Castrate	A	24	3	0	13	10. 1 ± 0. 49	10. 2 ± 0. 19
		C3H	24	5	1	25	14. 5 ± 1. 02	13. 8 ± 0. 80
		129	28	0	1	4	12. 1	18. 0 ± 1. 12
MCA thread for 4 weeks, then 33 percent diethylstilbestrol pellet‡	Castrate	A	20	15	3	90	6. 1 ± 0. 38	6. 1 ± 0. 35
		C3H	20	17	0	85	7. 2 ± 0. 31	7. 0 ± 0. 31
		129	21	20	0	95	6. 9 ± 0. 32	6. 7 ± 0. 32
Plain thread for 4 weeks, then 33 percent diethylstilbestrol pellet‡	Castrate	A	24	0	1	4	7. 6	5. 7 ± 0. 30
		A§	21	0	0			4. 6 ± 0. 27
		C3H	19	3	1	21	14. 5 ± 1. 07	13. 6 ± 0. 84
		129	20	0	1	5	4. 2	3. 8 ± 0. 18
Plain thread for 4 weeks	Castrate	A	24	0	0			10. 6 ± 0. 41
		A§	24	0	0			10. 3 ± 0. 92
		C3H	21	0	0			12. 0 ± 1. 29
		129	20	0	0			15. 3 ± 1. 61

*Average dimension of tumors as measured in sections.
†Standard error of the mean.
‡Diethylstilbestrol-cholesterol pellets weighed 5 mg and were inserted subcutaneously.
§Supracervical hysterectomy at time of castration.

C3HeB castrates at an average latent period of 14.5 ± 1.02 months. One of the smaller carcinomas observed at 13.9 months is illustrated in figures 16 and 17. Among the strain 129 castrates, 1 microscopic invasive cervical lesion, measuring 0.4 mm in average dimension, was seen at 12.0 months (fig. 24). A representative group of the smallest invasive lesions tabulated in these experiments (figs. 24 to 27) is assembled out of sequence on a single plate. No other tumors of the reproductive tract occurred among these animals.

Methylcholanthrene-coated thread, then diethylstilbestrol pellet.—The alternate castrates among the three strains that had received 33 percent diethylstilbestrol pellets at the time of thread removal developed cervical carcinoma at an incidence level of between 85 percent for strain C3HeB and 95 percent for strain 129, at average latent periods approaching those of the continuous thread experiments (text-fig. 5, table 4).

Among 20 strain A females, 15 larger and 3 microscopic cervical carcinomas were observed at an average latent period of 6.1 ± 0.38 months. The smallest invasive lesion is illustrated in figure 25. Among 20 strain C3HeB females, 17 cervical carcinomas were observed at an average latent period of 7.2 ± 0.31 months. The smallest tumor measured 2.5 mm in average dimension in the sections. A sarcoma of the cervix was associated with one carcinoma in this group. Among 21 strain 129 females, 20 cervical carcinomas, all greater than 1.5 mm in average dimension, were observed at an average latent period of 6.9 ± 0.32 months.

The toxic effects of the large dose of estrogen, including pyometra, pelvic abscesses, and bladder obstruction, along with the induced tumors, contributed to a reduction of the lifespan of these animals. However, the large size of the cervical carcinomas together with their high incidence demonstrates a major new incidence of tumors, which do not appear during the lifespan of either carcinogen or diethylstilbestrol-treated castrates.

Plain thread, then diethylstilbestrol pellet.—The possible carcinogenic role of the 33 percent diethylstilbestrol pellets was investigated by parallel experiments substituting plain threads for methylcholanthrene-coated threads. The castrate endocervical epithelium was stimulated for 4 weeks by an uncoated silk thread followed by subcutaneous insertion of a diethylstilbestrol pellet. An attempt was made in strain A mice to diminish the role of pyometra as a contributing cause of death by a supracervical hysterectomy at the time of castration. The results are summarized in text-figure 6 and table 4.

One microscopic carcinoma of the cervix occurred at 7.6 months among 24 strain A castrates bearing diethylstilbestrol pellets. No carcinomas occurred among 21 strain A castrates that had been subjected to supracervical hysterectomy. Hysterectomy produced no improvement in average survival in this strain. Four cervical carcinomas were observed among 19 strain C3HeB pellet-bearing castrates at an average latent period of 14.5 ± 1.07 months. A carcinoma of the endocervical canal, measuring 1.6 mm in average dimension, was demonstrated at 17.5 months (figs. 18 and 19). The smallest carcinoma, observed at 14.1 months, was

located in the upper vagina and was associated with a papilloma of the vulva. The strain C3HeB mice bearing 33 percent dithylstilbestrol pellets showed no shortening of average survival as compared with alternate plain-thread controls. Strain 129 mice appeared unusually sensitive to the toxic effects of 33 percent diethylstilbestrol pellets as compared with their alternate plain-thread controls (table 4). One microscopic cervical carcinoma appeared at 4.2 months among 20 strain 129 mice. In addition, 2 papillary tumors of the vulva occurred in this strain.

The incidence of 6 carcinomas among 84 mice, 7 percent, when compared with the 85 to 95 percent incidence of cervical carcinoma among 61 mice of the same three strains in which estrogen treatment had been preceded by a 4-week exposure to methylcholanthrene-coated threads (text-fig. 5), indicates a minor role for estrogen alone in the induction of cervical carcinoma within the latent period of these experiments.

Plain thread for 4 weeks in castrates.—No cervical carcinomas appeared among 89 castrates exposed to the stimulation of uncoated endocervical threads for 4 weeks (text-fig. 6, table 4). Thus there was no evidence of spontaneous cervical carcinogenesis or of a nonspecific carcinogenic response to the irritation of the thread in these experiments. The survival data on the castrates of the three strains provide a base line for comparison with the survival of animals exposed to methylcholanthrene or to estrogen.

Thread in Castrates 4 Weeks, Then 10 percent Estrogen Pellet

The effect of a substantially lower dose of estrogen in these experiments was investigated in strains A, C3HeB, and 129. Ten percent diethylstilbestrol-cholesterol pellets were selected as providing a dose that might be expected to have only a slight effect on survival time while maintaining a high level of estrogenic stimulation for at least a year (*20, 21*). Females, castrated at 2 to 3 months of age, after a rest period of 4 weeks received methylcholanthrene-coated endocervical threads which were removed after 4 weeks. When the threads were removed, half of the strain A castrates and alternate groups of strains C3HeB and 129 castrates received 10 percent diethylstilbestrol-cholesterol pellets subcutaneously.

Methylcholanthrene-coated thread alone.—The baseline incidence of cervical carcinoma in castrates, which received methylcholanthrene-coated threads for 4 weeks but no estrogen treatment, ranged from 0 in strain A to 5 percent in strain C3HeB (text-fig. 7, table 5).

No cervical carcinomas occurred among 25 strain A castrates. One sarcoma of the cervix was observed at 13.0 months. Two cervical carcinomas occurred among 37 strain C3HeB castrates. One cervical carcinoma occurred at 4.4 months among 28 strain 129 castrates. Two papillary tumors of the vulva were observed in this strain.

The incidence of cervical carcinoma among castrates in this group of experiments was somewhat lower than in the preceding control experiments (table 4). The average survival of each strain showed no significant difference in the two experiments and emphasized the order

TABLE 5.—Methylcholanthrene-coated and plain endocervical threads for 4 weeks in castrates with and without subsequent *10 percent diethylstilbestrol pellets:* incidence and latent period of squamous-cell carcinoma and survival

Procedure	Gonadal status	Strain	Number of mice	Squamous-cell carcinoma >1.5 mm	Squamous-cell carcinoma <1.5 mm*	Percent carcinoma	Average latent period carcinoma (months ± SE)†	Average survival (months ± SE)†
MCA thread for 4 weeks	Castrate	A	25	0	0			10.3 ± 1.08
		C3H	37	1	1	5	12.9 ± 3.56	13.1 ± 0.85
		129	28	1	0	4	4.4	16.5 ± 1.23
MCA thread for 4 weeks, then 10 percent diethylstilbestrol pellet‡	Castrate	A	23	2	3	22	13.7 ± 1.70	10.9 ± 1.01
		C3H	37	6	1	19	8.8 ± 2.53	10.5 ± 0.82
		129	34	5	1	18	10.9 ± 1.40	12.0 ± 0.84
Plain thread for 4 weeks, then 10 percent diethylstilbestrol pellet‡	Castrate	A	16	0	1	6	17.6	12.4 ± 1.33
		A§	49	0	0			12.5 ± 0.65

*Average dimension of tumors as measured in sections.
†Standard error of the mean.
‡Diethylstilbestrol-cholesterol pellets weighed 5 mg and were inserted subcutaneously.
§Supracervical hysterectomy at time of castration.

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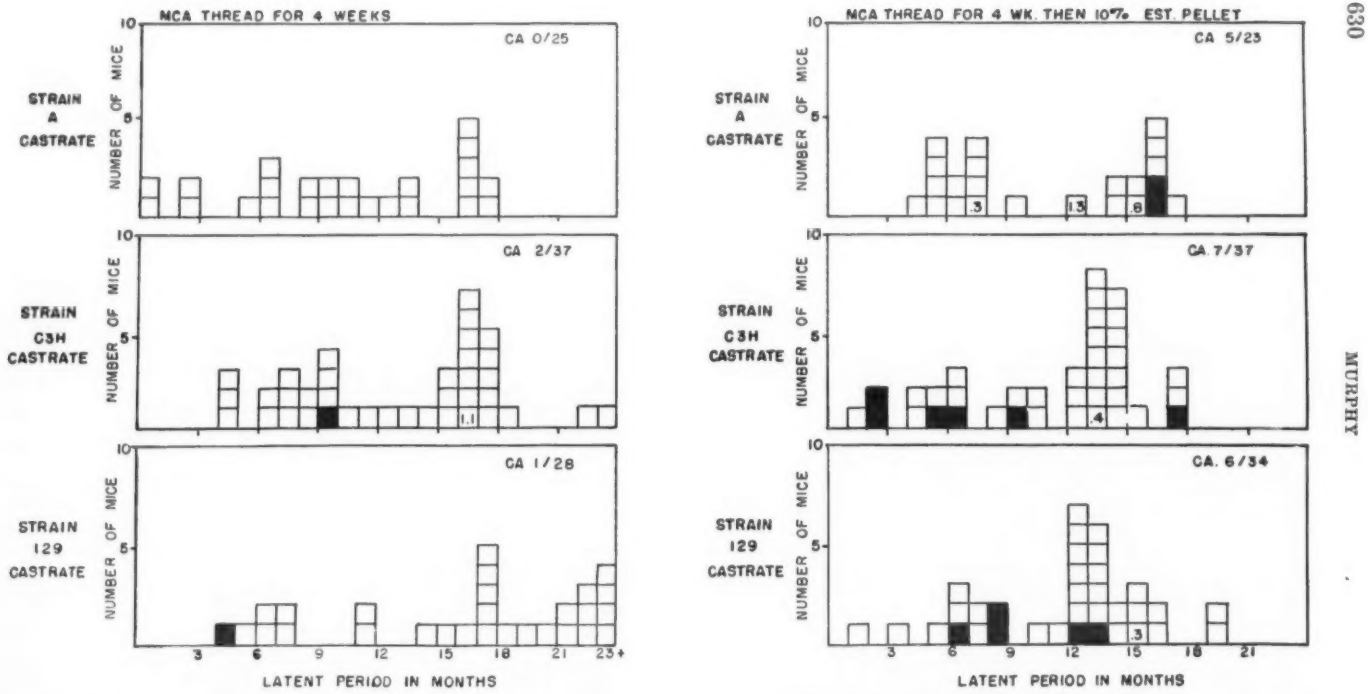


TEXT-FIGURE 7.—MCA THREAD FOR 4 WEEKS IN CASTRATES. Incidence and latent period of squamous-cell carcinoma of the cervix and survival in castrates that had received methylcholanthrene-coated thread left in the endocervical canal for 4 weeks. Half of the animals in addition received a 10 percent diethylstilbestrol pellet subcutaneously at the time of thread removal.

of increasing longevity of female castrates among the inbred strains A, C3HeB, and 129.

Methylcholanthrene-coated thread, then diethylstilbestrol pellet.—Ten percent diethylstilbestrol-cholesterol pellets after a 4-week exposure to an endocervical thread coated with methylcholanthrene increased the incidence of squamous-cell carcinoma: in strain A from 0 to 22 percent, in strain C3HeB from 5 to 19 percent, and in strain 129 from 4 to 18 percent (text-fig. 7, table 5). The average survival time of castrates was not affected by 10 percent diethylstilbestrol pellets in strain A and appeared only moderately reduced in strains C3HeB and 129 (table 5).

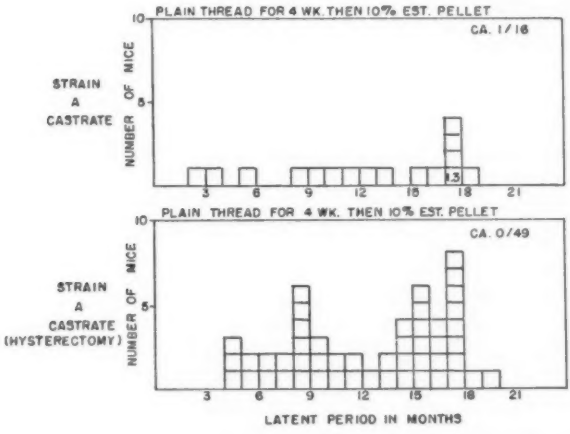
Five squamous-cell carcinomas occurred among 23 strain A females at an average latent period of 13.7 ± 1.70 months. Three were microscopic carcinomas ranging in average dimension from 0.3 to 1.3 mm. Seven cervical carcinomas occurred among 37 C3HeB females at an average latent period of 8.8 ± 2.53 months. The largest carcinoma measured 10 mm in average dimension (figs. 20 and 21). The only microscopic lesion in this group measured 0.4 mm in average dimension and is illustrated in figure 26. Six cervical carcinomas occurred among 34 strain 129 females at an average latent period of 10.9 ± 1.40 months. The only microscopic lesion in this group measured 0.3 mm in average diameter and is illustrated in figure 27. This is the smallest invasive lesion tabulated in these experiments.

An unusual number and variety of genital-tract tumors other than squamous-cell carcinoma occurred among the longer survivors in this group of experiments, particularly in strain 129. In strain A, a granular-cell "myoblastoma" of the cervix accompanying a cervical carcinoma occurred at 16.2 months. In addition, a papillary tumor of the vulva was found at 16.2 months. In strain C3HeB, 4 sarcomas primarily involving the cervical region were observed at an average latent period of 10.3 months and 1 papillary tumor of the vulva at 13.2 months. In strain 129, there were 6 sarcomas of the cervical region, observed at an average latent period of 13.4 months. Eight papillary tumors of the vulva, a number of them showing invasive properties, occurred at an average latent period of 12.0 months. In addition, there were 2 adenocarcinomas of the endometrium at the lower end of a uterine horn and 1 hemangioendothelioma of the cervix.

Plain thread, then diethylstilbestrol pellet.—The carcinogenic role of 10 percent diethylstilbestrol-cholesterol pellets in these experiments was investigated for strain A castrates by stimulation of the endocervical epithelium for 4 weeks with plain silk thread, after which the diethylstilbestrol pellet was inserted subcutaneously (text-fig. 8, table 5). In one experiment, a microscopic cervical carcinoma measuring 1.3 mm in average dimension occurred at 17.6 months among 16 strain A castrates (figs. 22 and 23). In a second experiment, no carcinomas occurred among 49 strain A castrates, which had also been subjected to supracervical hysterectomy at the time of castration. There was no significant difference in average survival between these two groups. Their average

survival lies within the range of survival of the strain A/Sn castrates of all the groups that had not received 33 percent diethylstilbestrol pellets (tables 4 and 5).

Maximal estrogen stimulation of the stratified squamous epithelium of the cervix and vagina was maintained for over 9 months by 10 percent diethylstilbestrol-cholesterol pellets, as judged by the number of layers of epithelial cells and the degree of surface keratinization. A lesser degree of keratinization characterized the longer survivors, with some diminution in average thickness of the epithelium appearing in scattered fashion among survivors up to 20 months. Thus 10 percent diethyl-



TEXT-FIGURE 8.—PLAIN THREAD FOR 4 WEEKS IN CASTRATES, THEN 10 PERCENT DIETHYLSTILBESTROL PELLET. Incidence and latent period of squamous-cell carcinoma of the cervix and survival in castrate strain A mice that had received uncoated endocervical thread for 4 weeks, then a 10 percent diethylstilbestrol pellet inserted subcutaneously at time of thread removal. *Lower panel* represents animals subjected to supracervical hysterectomy at time of castration.

stilbestrol pellets maintained a high level of estrogenic stimulation during the latent periods of these experiments.

It can be concluded that 10 percent diethylstilbestrol pellets produce a significant increase in the incidence of squamous-cell carcinoma of the cervix when they follow limited exposure of the endocervical epithelium in castrates to a methylcholanthrene-coated thread.

DISCUSSION

Spontaneous carcinoma of the cervix, a major cause of death from cancer among women, occurs rarely in laboratory mice or in any other mammal (*22*). However, the capacity of the mouse to develop spontaneous carcinoma of the cervix and vagina was established by Gardner

and Pan in studies on an inbred line derived from the Pybus-Miller strain (*23*). They described 12 spontaneous carcinomas of the cervix, vaginal fornices, and upper vagina among 56 mice. Unfortunately, the strain was lost because of a high incidence of associated sterility. The inbred mouse has become the laboratory animal most extensively used in studies on cervical carcinogenesis. Cervical and vaginal carcinomas have been induced either by systemic administration of estrogenic hormones or by the local application of carcinogens. Interrelationships between these two methods of inducing carcinoma have received limited emphasis.

In comparative studies on cervical carcinogenesis, what justification is there for emphasis on the cervical epithelium of the mouse? In terms of human disease, squamous-cell carcinoma of the cervix is a common problem, while carcinoma of the vagina is a rarity. Schiller postulated the squamocolumnar junction as the common site of origin of human cervical carcinoma (*24*). Fidler and Boyd (*25*) have reported the squamocolumnar junction and the endocervical canal as the predominant sites of early invasion. In women the unstable squamocolumnar junction is located near the external os of the cervix. In the bicornuate uterus of the mouse the junctions are just above the bifurcation of the endocervical canal. The stratified squamous epithelium of the endocervical canal is less responsive to cyclic hormonal stimulus than the epithelium of the vagina (*26*). Possibly such factors and other anatomical and physiological factors may determine localization of the carcinogenic stimulus and susceptibility to carcinogenesis of the stratified squamous epithelium of the human female reproductive tract. An experimental approach to the problems of human cervical carcinogenesis should encompass such factors wherever practical.

Blind introduction of chemical carcinogens into the vagina usually produces tumors of the vagina and vulva rather than of the cervix (*5*, *11*, *27*). Painting the cervix with a carcinogen dissolved in a highly volatile solvent, particularly when done under visual control, can concentrate the maximal exposure to the ectocervix, the vaginal fornices, and the upper vagina, and minimize the induction of tumors of the lower vagina and vulva. Scarpelli and von Haam (*22*) analyzed the localization of 139 carcinomas induced by painting the cervix with benzo[*a*]pyrene dissolved in acetone, applied by means of a wire loop. They report that 19.5 percent of the tumors were primary in the vagina, 41.0 percent on the cervix, and that 39.5 percent involved both cervix and vagina. A comparable distribution was found in the experiments reported here. It appears unlikely that further refinements in the cervical-painting technique can be expected to produce high yields of cervical carcinoma without significant admixture with carcinoma of the vagina. The carcinomas produced by prolonged treatment with estrogen alone arise from both the ectocervix and the upper vagina, presenting a similar admixture of vaginal and cervical tumors that can be distinguished only in their microscopic stages. In this instance the separation of the two sites of origin may be less important, since both vaginal and cervical tumors arise in an

epithelium, which shows a diminished response to estrogen after prolonged treatment (*8*, *9*).

The endocervical-thread technique effectively restricts the exposure to carcinogen to the stratified squamous epithelium of the endocervical canal. The stimulus was sufficiently intense to produce yields of cervical carcinoma as high as 78 percent and average latent periods as short as 5 months, for tumors advanced enough to cause death. It is probable that further intensification of the carcinogenic stimulus, together with better control of the infectious processes stimulated by a foreign body, would give yields approaching 100 percent cervical carcinoma, with even shorter latent periods. A more important problem concerns better quantifying of the initial carcinogenic stimulus. The precision of a single initiating application of carcinogen that has been employed in the field of skin carcinogenesis does not as yet appear practical in the field of cervical carcinogenesis. Actually, a single painting of the skin probably involves a prolonged exposure of epithelial cells to a carcinogen which is soluble in sebum and enters the accessory sebaceous glands. Quantifying by limiting the duration of exposure to a carcinogen appears to hold the greatest promise in the experimental approach to the carcinogenesis of an internal stratified squamous epithelium that has no accessory glands.

The epithelium of castrates was selected as the baseline for studies on the effects of added estrogenic hormone. Painting and continuous-thread experiments had indicated that the epithelium of castrates was no less responsive to carcinogenesis by methylcholanthrene than the epithelium of females undergoing estrous cycles. Evidence for increased responsiveness of the castrate epithelium to 4-week exposure to a methylcholanthrene-coated thread was found in strain C3H (text-fig. 4). Three carcinomas appeared among castrates during a 14-month period, while none appeared among intact animals. In the experiments involving subsequent administration of 33 and 10 percent diethylstilbestrol pellets, the castrates treated with methylcholanthrene alone gave a low comparative incidence of cervical carcinoma, suitable for the analysis of promoting factors.

The strain differences in response to the experimental treatments primarily concerned sensitivity to the toxic effects of estrogen treatment. The order of sensitivity of the inbred strains to the toxic effects of 33 percent diethylstilbestrol pellets was 129, A, and C3H, as measured by shortening of survival time. Ten percent pellets produced only a slight shortening of average survival in strains 129 and C3H, even though carcinoma occurred in approximately 20 percent of these animals. There were no significant strain differences in the incidence of carcinoma when 33 and 10 percent diethylstilbestrol pellets followed 4-week exposure to methylcholanthrene. In the painting experiments the incidence of carcinoma was lower in C57BL than in A. However, C57BL mice tolerated the painting procedure poorly and their survival time was greatly shortened by the development of pelvic abscesses and rectovaginal fistulas. Of the strains studied, C3H without the mammary-tumor agent offers a

number of advantages. It is characterized by long survival, high resistance to the toxic effects of estrogen, and good tumor yields.

The design of these experiments, in which limited exposure to a methylcholanthrene-coated thread was followed by systemic administration of a synthetic estrogen, lends itself to analysis in terms of "initiation" and "promotion" (*28*). It must be recognized, of course, that the 4-week exposure to methylcholanthrene permitted both the initiating, and to some extent, the promoting effects of the compound to be expressed. The sequence of methylcholanthrene and 33 percent diethylstilbestrol produced a much higher proportion of tumors, a total of 55 carcinomas among 61 mice or 90.2 percent in the three strains, than the sum of the tumors produced by the two agents acting alone. There were only 10 carcinomas among 76 mice or 13.2 percent for methylcholanthrene alone, and 6 carcinomas among 84 mice or 7.1 percent for estrogen alone. In these terms a striking promoting effect of estrogen can be postulated.

The promoting effect of 10 percent diethylstilbestrol pellets is significant but less intense than that of 33 percent pellets. In strain A, the sequence of methylcholanthrene and 10 percent diethylstilbestrol yielded tumors in 5 of 23 mice or 21.7 percent. Methylcholanthrene alone gave no tumors among 25 animals. Estrogen alone gave 1 tumor in 65 animals or 1.5 percent. The three strains can be combined to give an incidence of 3 tumors among 90 animals exposed to methylcholanthrene alone, or 3.3 percent. Eighteen tumors appeared among 94 animals receiving the sequence of agents, an incidence of 19.1 percent. The 10 percent pellets were much less toxic than the 33 percent pellets and the lifespan was only slightly reduced. Part of this reduction can be attributed to the tumors that were induced.

Can the promoting effect of estrogen be explained in terms of a simple increase in mitotic activity, producing perhaps many more daughter cells than might be produced during the normal cyclic activity of the entire lifespan of the mouse? It might be postulated that such an increase in the number of daughter cells derived from cells initially altered by exposure to the carcinogen would increase the opportunity for the occurrence of additional changes required to produce a carcinoma. However, maximal estrogenic stimulation of the stratified squamous epithelium of the cervix and vagina was maintained for over 9 months by 10 percent diethylstilbestrol pellets, as judged by the number of layers of epithelial cells and the degree of surface keratinization. The level of epithelial stimulation was comparable to that produced by 33 percent pellets during this period. It can be concluded that both 33 and 10 percent pellets produced a high level of mitotic stimulation during the period of tumor induction. Yet the difference in tumor incidence with the two doses of estrogen, 90.2 percent as compared with 19.1 percent, suggests the operation of factors other than mitotic turnover. It is possible that toxic levels of estrogen may produce additional alterations in cellular metabolism in a process initiated by methylcholanthrene.

Dao and Sunderland (*29*) summarized the evidence for a promoting

effect of progesterone in mammary carcinogenesis in rats and mice when the initiating stimulus is a systemically administered chemical carcinogen. Cherry and Glucksmann (*27*) reported the effects of endocrine changes on the induction of vaginal tumors in rats by painting with 7, 12-dimethylbenz[*a*]anthracene. Castration reduced the incidence of tumors, and administration of estrogen or of progesterone raised the incidence of tumors only slightly. However, the majority of the tumors were sarcomas arising in the subepithelial stroma. Epithelial tumors occurred only rarely.

The possible role of uterine infection, which occurs commonly during continuous estrogen stimulation, must be considered when the promoting effect of estrogen treatment is assessed. Menkin (*30*) has proposed a cocarcinogenic role for inflammatory exudates in carcinogenesis. Weinstein *et al.* (*31*) have shown that the contents of the uterine lumen rapidly become infected with bacteria under conditions of estrogen treatment. Some degree of endometritis or pyometra was common in the animals which received 33 percent diethylstilbestrol pellets, and occurred, but less frequently, in the animals which received 10 percent pellets. Could the continued flow of purulent material through the endocervical canal contribute to the process of carcinoma development? Supracervical hysterectomy was performed at the time of castration in groups of strain A mice receiving 4-week exposure to a plain thread followed by 33 and 10 percent diethylstilbestrol pellets, in an attempt to increase the average survival. No improvement in survival time was observed. However, 2 microscopic carcinomas occurred among 32 simple castrates, while no carcinomas developed among 70 castrates that had also undergone supracervical hysterectomy. Thus, it is possible that inflammatory exudate may play a cocarcinogenic role in the induction of cervical and vaginal carcinoma by estrogen alone. On the other hand, under conditions of 4-week exposure to a methylcholanthrene-coated thread followed by 33 percent diethylstilbestrol pellets there was no reduction in incidence of carcinoma in a group of castrates which had been subjected to supracervical hysterectomy (unpublished data).

Are there any possible extrapolations from these experiments on the mouse to the problem of human cervical carcinoma? The experiment with breeding animals did not indicate a promoting effect of pregnancy in the mouse on the development of cervical carcinoma after limited exposure to a carcinogen. However, the demonstration that estrogen can have a promoting effect in the development of squamous-cell carcinoma of the epithelium of the female reproductive tract in the mouse suggests that it might be profitable to apply this concept of hormonal promotion to the analysis of the human disease. There has been no convincing evidence that estrogen imbalance alone can account for both the initiation and development of human cervical carcinoma. However, if the human disease involves an initiating stimulus such as smegma (*32*) or some other factor associated with the marital status or pregnancy (*33*, *34*), then it is possible that the hormonal changes of pregnancy, the common ovarian

functional disturbances, or administered hormones may provide an important promoting stimulus. This experimental design is adapted to testing interrelationships among the many extrinsic and intrinsic factors that have been postulated for the development of the human disease, such as smegma, the male ejaculate, contraceptive materials, trauma, chronic irritation, early mating, pregnancy and parity, race and heredity, and hormonal imbalance.

REFERENCES

(1) Cioli, R.: Tumori sperimentali da catrame dell'utero e loro sviluppo in rapporto alla funzione ovarica. Bull. e atti d. r. Accad. med. di Roma 55: 291–296, 1929.

(2) Fusco, G.: Cancro sperimentale dell'utero e della vagina. Arch. di ostet. e ginec. 19: 315–344, 467–469, 1932.

(3) Dobrovolskaia-Zavadskaia, N., and Simsilevich, F.: Au sujet de la réaction des organes génitaux de la souris aux instillations du 1:2:5:6 dibenzanthracene dans le vagin. *In* Libro de Oro (dedicado al Prof. Angel H. Roffo). Buenos Aires, 1935, pp. 293–301.

(4) Klenitzky, J. S.: On experimental cancer of the uterine cervix. Bull. biol. et med. exper. URSS 9: 3–6, 1940.

(5) Gardner, W. U.: Discussion. *In* Conference on Gastric Cancer. J. Nat. Cancer Inst. 1: 502–504, 1941.

(6) Lacassagne, A.: Tumeurs malignes, apparues au cours d'un traitement hormonal combiné, chez des souris appartenant à des lignées refractaires au cancer spontané. Compt. rend. Soc. biol. 121: 607–609, 1936.

(7) Loeb, L. Burns, E. L., Suntzeff, V., and Moskop, M.: Carcinoma-like proliferations in vagina, cervix, and uterus of mouse treated with estrogenic hormones. Proc. Soc. Exper. Biol. & Med. 35: 320–322, 1936.

(8) Gardner, W. U., and Allen, E.: Malignant and non-malignant uterine and vaginal lesions in mice receiving estrogens and androgens simultaneously. Yale J. Biol. & Med. 12: 213–234, 1939.

(9) Gardner, W. U.: Carcinoma of the uterine cervix and upper vagina: induction under experimental conditions in mice. Ann. New York Acad. Sc. 75: 543–564, 1959.

(10) ———: Experimental induction of uterine cervical and vaginal cancer in mice. Cancer Res. 19: 170–176, 1959.

(11) Fishman, M., Shear, M. J., Friedman, H. F., and Stewart, H. L.: Studies in carcinogenesis. XVII. Local effect of repeated application of 3,4-benzpyrene and of human smegma to the vagina and cervix of mice. J. Nat. Cancer Inst. 2: 361–367, 1942.

(12) Pan, S. C., and Gardner, W. U.: Induction of malignant tumors by methylcholanthrene in transplanted uterine cornua and cervices of mice. Cancer Res. 8: 613–621, 1948.

(13) Murphy, E. D.: Studies on carcinogen-induced carcinoma of the cervix in mice. Am. J. Path. (Abstract.) 29: 608, 1953.

(14) von Haam, E., and Menzies, P.: Experimental studies in exfoliative cytology. Proc. Am. Assoc. Cancer Res. (Abstract.) 1: 22, 1953.

(15) von Haam, E., and Scarpelli, D. G.: Experimental carcinoma of the cervix: a comparative cytologic and histologic study. Cancer Res. 15: 449–455, 1955.

(16) Reagan, J. W., Wentz, W. B., and Machicao, N.: Induced cancer of the cervix uteri in the mouse. A.M.A. Arch. Path. 60: 451–456, 1955.

(17) Vellios, F., and Griffin, J.: The pathogenesis of dimethylbenzanthracene-induced carcinoma of the cervix of rats. Cancer Res. 17: 364–366, 1957.

(*18*) MURPHY, E. D.: Increased incidence of cervical cancer in mice when stilbestrol pellets were administered following limited exposure to methylcholanthrene. Proc. Am. Assoc. Cancer Res. (Abstract.) 2: 234–235, 1957.

(*19*) ———: Experimental cervical carcinogenesis: the promoting effect of estrogen. Seventh Internat. Cancer Cong., Abstracts of papers, London, 1958, pp. 191–192.

(*20*) SHIMKIN, M. B., and WHITE, J.: Absorption rate of hormone-cholesterol pellets. Endocrinology 29: 1020–1025, 1941.

(*21*) SHIMKIN, M. B., GRADY, H. G., and ANDERVONT, H. B.: Induction of testicular tumors and other effects of stilbestrol-cholesterol pellets in strain C mice. J. Nat. Cancer Inst. 2: 65–80, 1941.

(*22*) SCARPELLI, D. G., and VON HAAM, E.: Experimental carcinoma of the uterine cervix. Progr. Exper. Tumor Res. 1: 179–224, 1960.

(*23*) GARDNER, W. U., and PAN, S. C.: Malignant tumors of the uterus and vagina in untreated mice of the PM stock. Cancer Res. 8: 241–256, 1948.

(*24*) SCHILLER, W.: Early diagnosis of carcinoma of the cervix. Surg. Gynec. & Obst. 56: 210–222, 1933.

(*25*) FIDLER, H. K., and BOYD, J. R.: Occult invasive squamous carcinoma of the cervix. Cancer 13: 764–771, 1960.

(*26*) ALLEN, E.: The oestrous cycle in the mouse. Am. J. Anat. 30: 297–371, 1922.

(*27*) CHERRY, C. P., and GLUCKSMANN, A.: The effect of endocrine changes, of irradiation and of additional treatment of the skin on the induction of tumours in the female genital tract of rats by chemical carcinogens. Brit. J. Cancer 14: 489–501, 1960.

(*28*) FRIEDEWALD, W. F., and ROUS, P.: The initiating and promoting elements in tumor production; an analysis of the effects of tar, benzpyrene, and methylcholanthrene on rabbit skin. J. Exper. Med. 80: 101–126, 1944.

(*29*) DAO, T. L., and SUNDERLAND, H.: Mammary carcinogenesis by 3-methylcholanthrene. I. Hormonal aspects in tumor induction and growth. J. Nat. Cancer Inst. 23: 567–585, 1959.

(*30*) MENKIN, V.: Biologic cocarcinogens. Progr. Exper. Tumor Res. 1: 279–310, 1960.

(*31*) WEINSTEIN, L., GARDNER, W. U., and ALLEN, E.: The effect of estrogenic hormones on the bacterial content of the uterus. Yale J. Biol. & Med. 16: 43–51, 1943.

(*32*) HEINS, H. C., DENNIS, E. J., and PRATT-THOMAS, H. R.: The possible role of smegma in carcinoma of the cervix. Am. J. Obst. & Gynec. 76: 726–735, 1958.

(*33*) CLEMMESEN, J.: On the etiology of some human cancers. J. Nat. Cancer Inst. 12: 1–21, 1951.

(*34*) STERN, E., and DIXON, W. J.: Cancer of the cervix—a biometric approach to etiology. Cancer 14: 153–160, 1961.

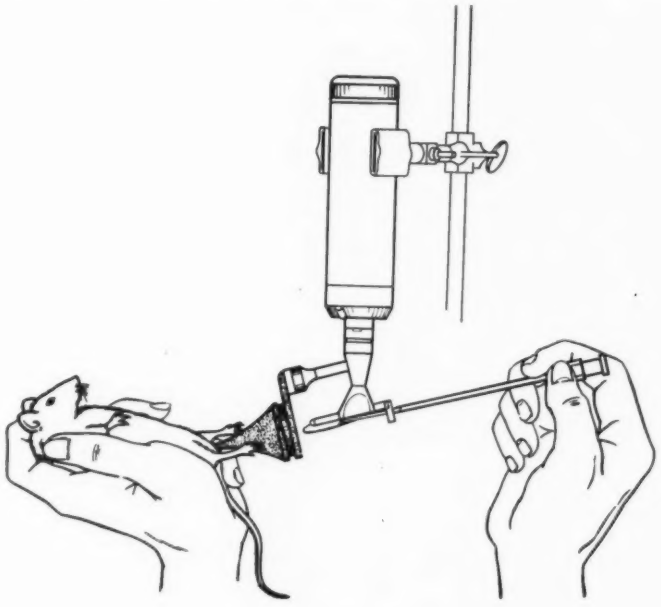
PLATES

600915—61——17

PLATE 76

FIGURE 1.—Method of visualizing and painting the cervix by means of an operating otoscope and infant-sized otic speculum.

FIGURE 2.—Position of methylcholanthrene-coated thread in endocervical canal. Coated segment extends from external os past squamocolumnar junction situated above internal bifurcation of endocervical canal and into lower portion of lumen of right uterine horn. (Drawing adapted from *The Biology of the Laboratory Mouse*, edited by G. D. Snell, Dover Publications, Inc., New York.)



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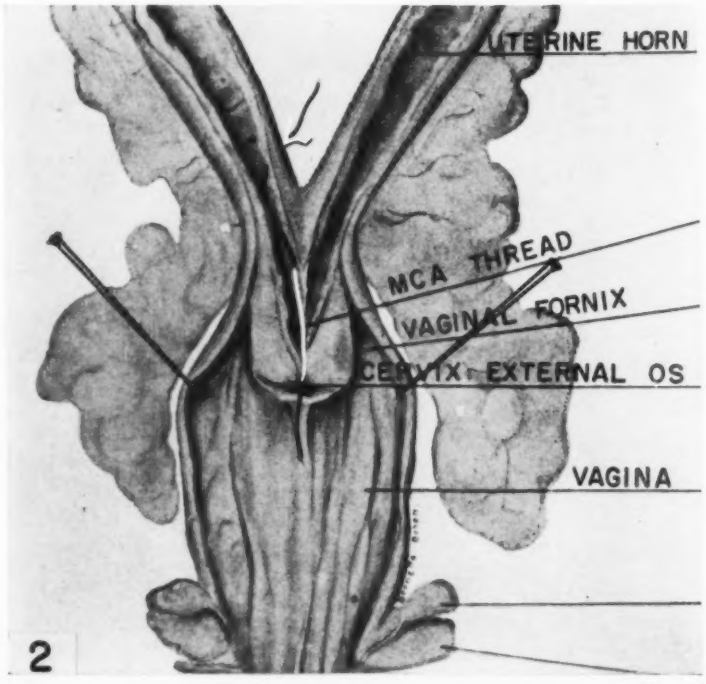

UTERINE HORN
MCA THREAD
VAGINAL FORNIX
CERVIX EXTERNAL OS
VAGINA

2

PLATE 77

Continuous methylcholanthrene-coated thread

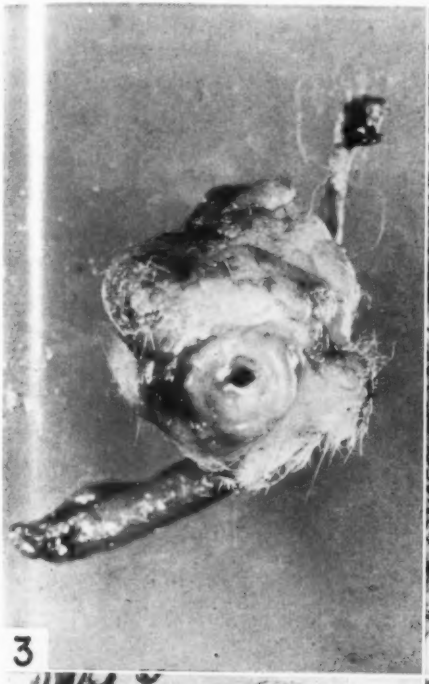
FIGURE 3.—Cervical carcinoma induced by continuous exposure to methylcholanthrene-coated endocervical thread in strain A/He castrated female, after 4.0 months. Vagina has been dissected away. Tumor has not invaded the epithelium covering the cervix. Tumor measured 6 mm in average diameter in histological sections and ranked 6th in size of 11 carcinomas among 14 mice. × 4

FIGURE 4.—Representative section of the above squamous-cell carcinoma of the cervix. × 150

Continuous MCA-coated thread and 33 percent diethylstilbestrol pellet

FIGURE 5.—Cervical carcinoma induced by continuous exposure to methylcholanthrene-coated endocervical thread in strain A/He castrate bearing 33 percent diethylstilbestrol pellet, after 5.8 months. Cross-section of cervix at upper limit of vaginal fornices. Tumor measured 3 mm in diameter, and ranked 3d in size of 6 carcinomas among 9 mice. × 16

FIGURE 6.—Higher magnification at periphery of squamous-cell carcinoma. × 150



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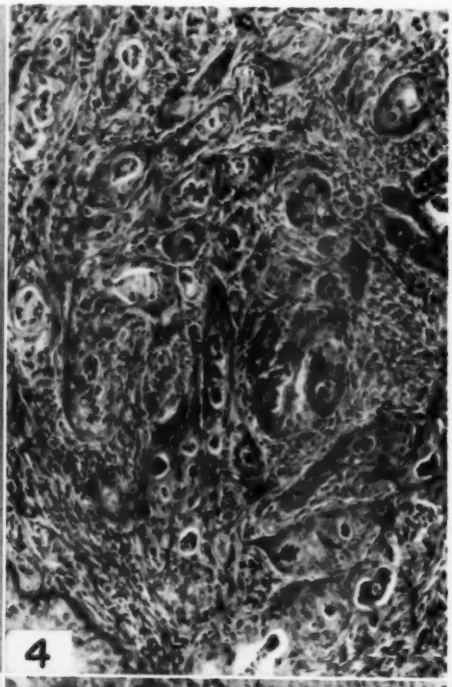

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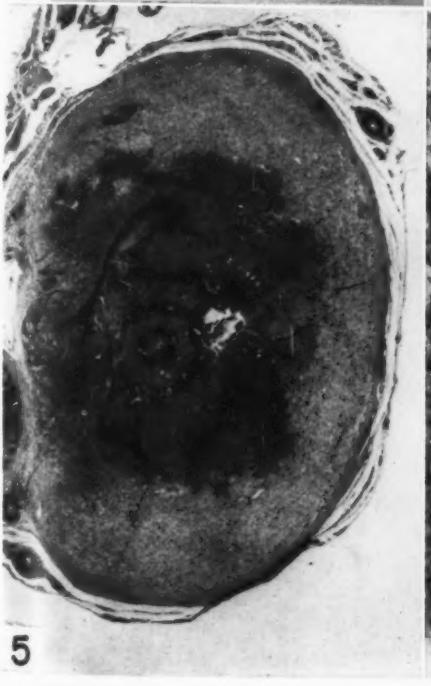

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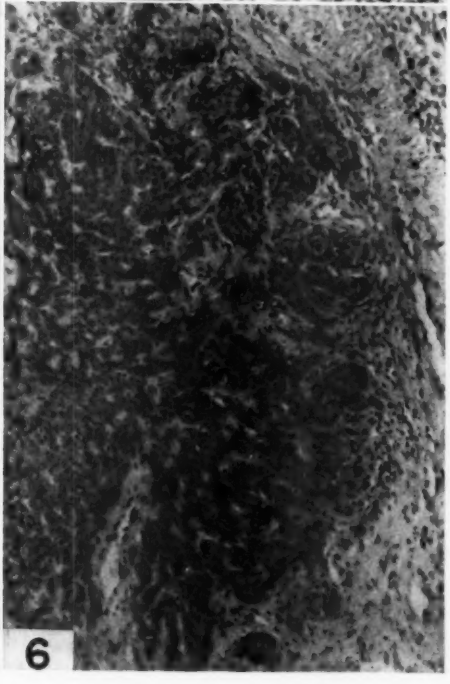

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PLATE 78

FIGURE 7.—Invasion of adjacent muscle wall of rectum by squamous-cell carcinoma of the cervix in strain A/Sn mouse. Primary tumor measured 6 mm in average dimension. × 60

FIGURE 8.—Invasion of wall of bladder by squamous-cell carcinoma in strain A/Sn mouse. Primary tumor measured 4 mm in average dimension. × 30

FIGURE 9.—Blood-vessel invasion at periphery of squamous-cell carcinoma of the cervix in strain C57BL/He mouse. Tumor measured 8 mm in average dimension. A tumor embolus was demonstrated in the lung. × 300

FIGURE 10.—Pulmonary metastasis of squamous-cell carcinoma of the cervix in strain A/He mouse. Tumor measured 10 mm in average dimension. × 200

FIGURE 11.—Section of 53d-generation transplant of cervical carcinoma induced in strain 129/J castrate by methylcholanthrene. × 75

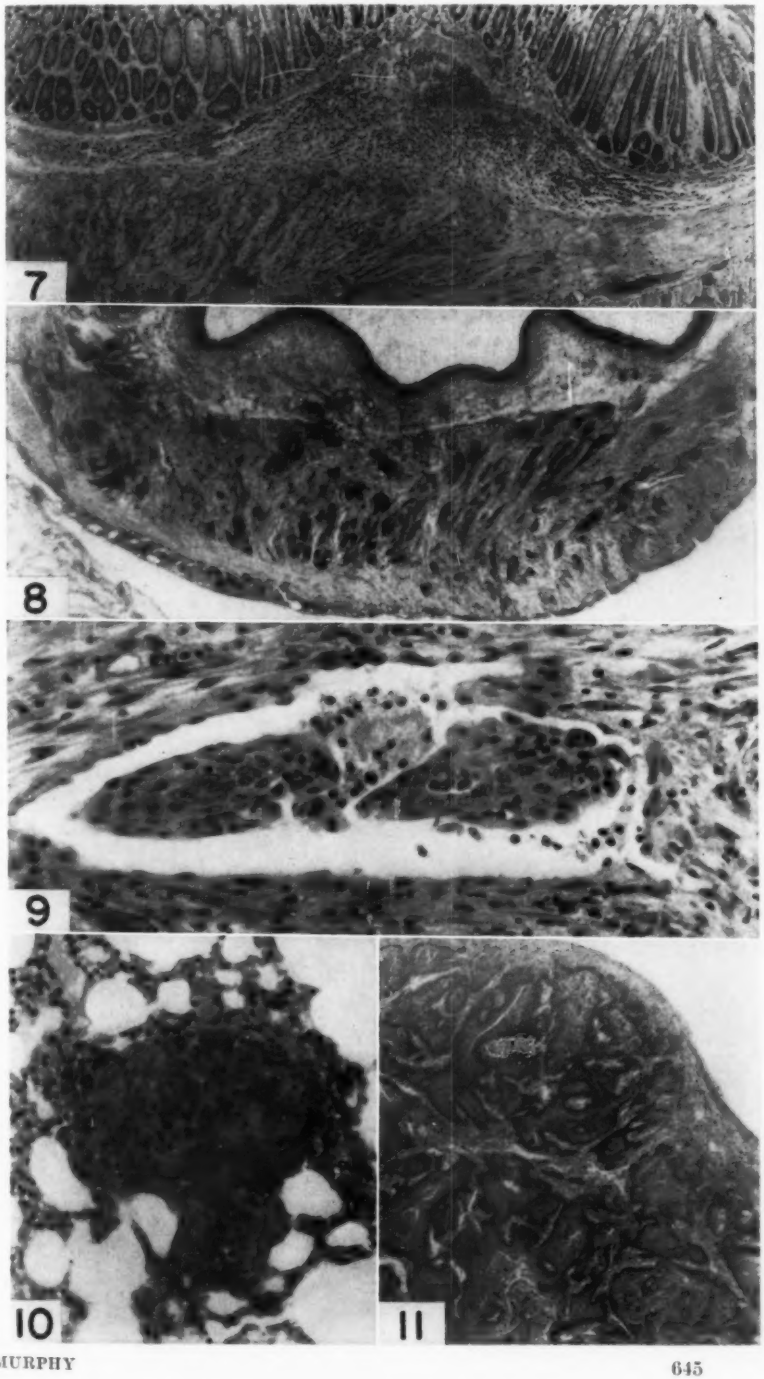
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PLATE 79

Continuous plain thread with 33 percent diethylstilbestrol pellet

FIGURE 12.—Cervical carcinoma induced in strain A/He castrate bearing 33 percent diethylstilbestrol pellet and uncoated endocervical thread, after 11.7 months. Cross-section of cervix near upper limit of vaginal fornices. Tumor measured 2.3 mm in diameter and was the only tumor observed among 9 mice. × 19

FIGURE 13.—Higher magnification at periphery of squamous-cell carcinoma. × 150

Methylcholanthrene-coated thread for 4 weeks, then 33 percent diethylstilbestrol pellet

FIGURE 14.—Cervical carcinoma induced in strain C3Hf/He castrate exposed to a methylcholanthrene-coated endocervical thread for 4 weeks followed by systemic exposure to 33 percent diethylstilbestrol pellet. Latent period was 3.2 months. Tumor measured 5.5 mm in average diameter, ranking 2d in size of 7 carcinomas appearing among 12 mice. × 12

FIGURE 15.—Higher magnification of squamous-cell carcinoma from region of endocervical canal. × 150

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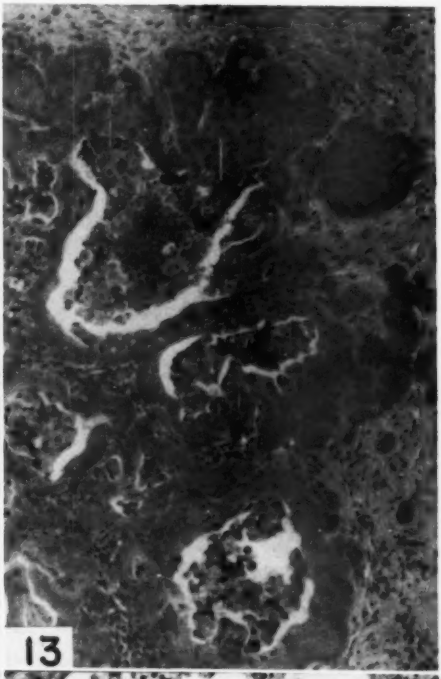
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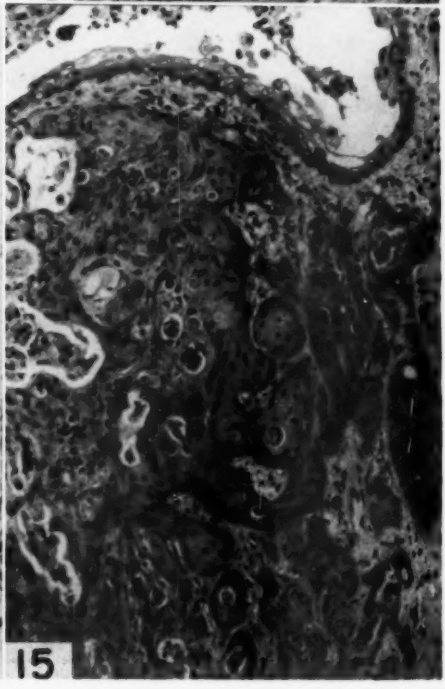
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PLATE 80

Methylcholanthrene-coated thread for 4 weeks

FIGURE 16.—Cervical carcinoma induced in strain C3HeB/FeJ castrate exposed to methylcholanthrene-coated endocervical thread for 4 weeks. Latent period was 13.9 months. Cross-section of cervix at level of vaginal fornices. Tumor measured 0.9 mm in average dimension and ranked 5th in size of 6 carcinomas appearing among 24 mice. × 36

FIGURE 17.—Higher magnification of glandular-appearing squamous-cell carcinoma. × 150

Plain thread for 4 weeks, then 33 percent diethylstilbestrol pellet

FIGURE 18.—Cervical carcinoma induced in strain C3HeB/FeJ castrate by 33 percent diethylstilbestrol pellet inserted subcutaneously at time of removal of plain endocervical thread. Cross-section of cervix near upper limit of vaginal fornices. Latent period was 17.5 months. Tumor measured 1.6 mm in average dimension, ranking 3d in size of 4 carcinomas appearing among 19 mice. × 16

FIGURE 19.—Higher magnification of squamous-cell carcinoma. × 150

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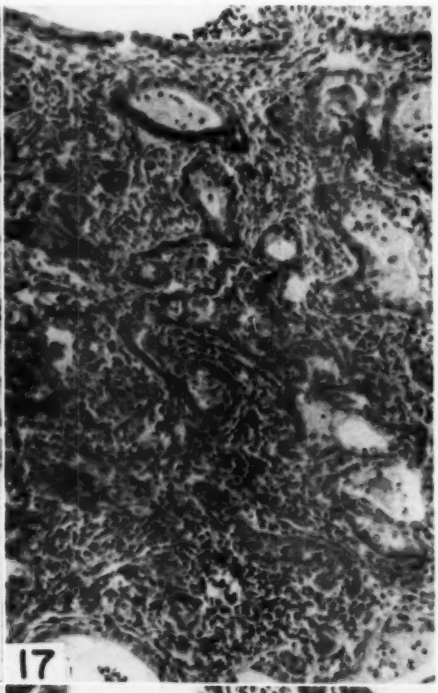
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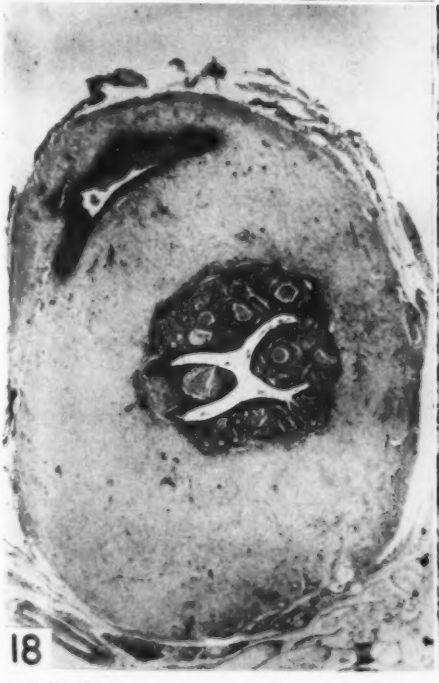
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PLATE 81

Methylcholanthrene-coated thread for 4 weeks, then 10 percent diethylstilbestrol pellet

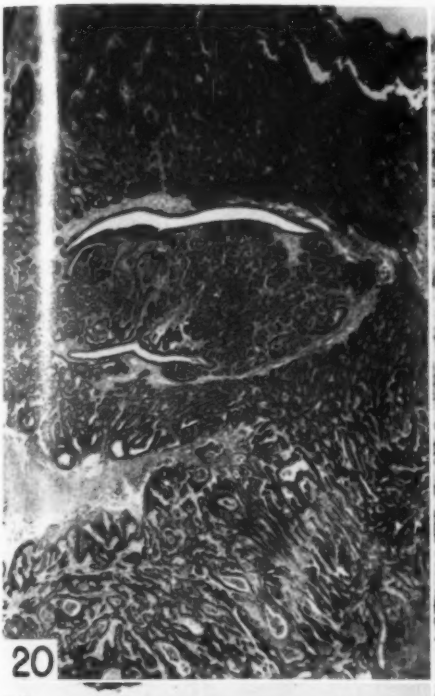
FIGURE 20.—Cervical carcinoma induced in strain C3HeB/FeJ castrate exposed to methylcholanthrene-coated endocervical thread for 4 weeks, followed by systemic exposure to 10 percent diethylstilbestrol pellet inserted subcutaneously at time of thread removal. Latent period was 7.0 months. Tumor measured 10 mm in average dimension, ranking largest in size of 7 carcinomas appearing among 37 mice. × 16

FIGURE 21.—Higher magnification of central portion of squamous-cell carcinoma. × 150

Plain thread for 4 weeks, then 10 percent diethylstilbestrol pellet

FIGURE 22.—Cervical carcinoma induced in strain A/Sn castrate by 10 percent diethylstilbestrol pellet inserted subcutaneously at time of removal of plain endocervical thread. Cross-section of cervix near upper limit of vaginal fornices. Latent period was 17.6 months. Tumor measured 1.3 mm in average dimension. This was the only carcinoma appearing among 16 mice. × 21

FIGURE 23.—Higher magnification of squamous-cell carcinoma. × 150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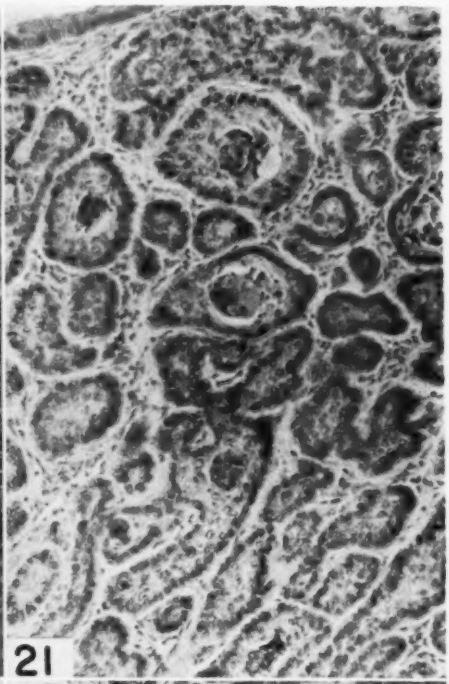
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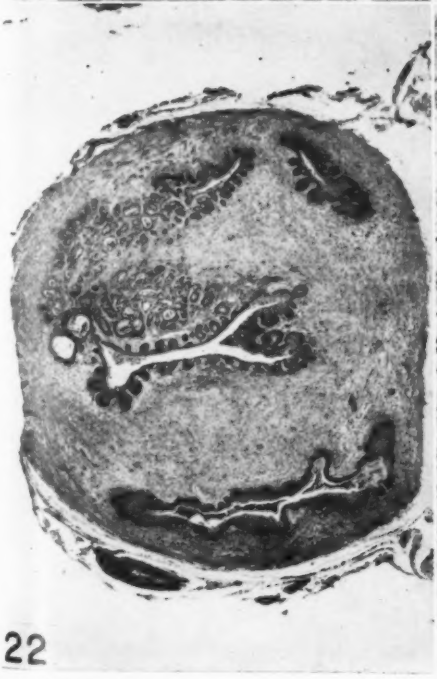
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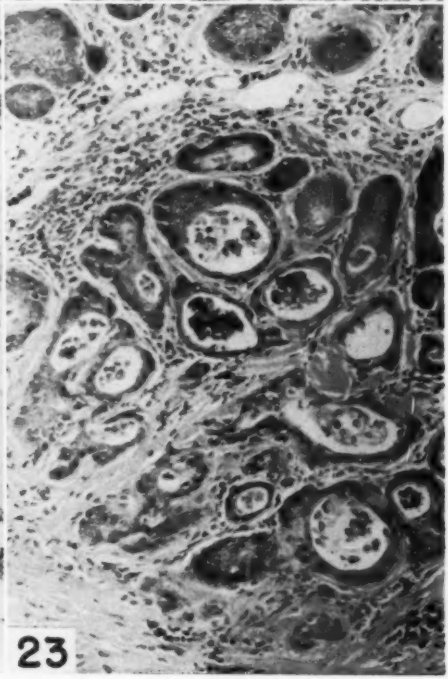
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PLATE 82

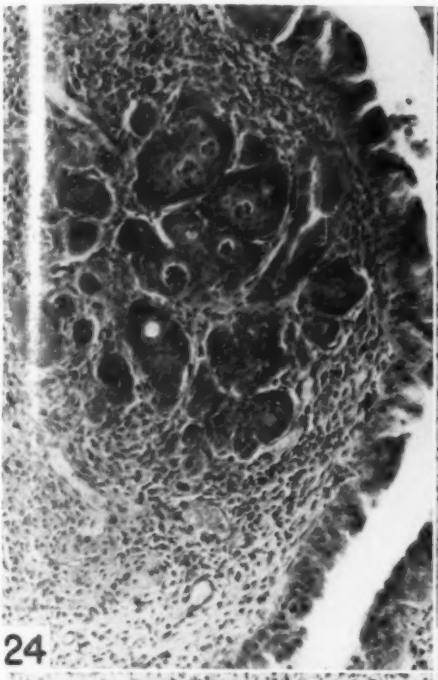
Representative microscopic carcinomas selected from smaller lesions included in data

FIGURE 24.—Microscopic carcinoma of endocervical canal near external os, induced in strain 129/J castrate exposed to a methylcholanthrene-coated endocervical thread for 4 weeks. Latent period was 12.0 months. Tumor measured 0.4 mm in average dimension and was the only carcinoma appearing among 28 mice. × 150

FIGURE 25.—Microscopic carcinoma of endocervical canal induced in strain A/J castrate exposed to methylcholanthrene-coated thread for 4 weeks, followed by systemic exposure to 33 percent diethylstilbestrol pellet. Latent period was 6.6 months. Tumor measured 0.4 mm in average dimension, ranking 18th in size of 18 carcinomas appearing among 20 mice. × 150

FIGURE 26.—Microscopic carcinoma of endocervical canal near external os induced in strain C3HeB/FeJ castrate exposed to a methylcholanthrene-coated thread for 4 weeks, followed by systemic exposure to a 10 percent diethylstilbestrol pellet. Latent period was 13.0 months. Tumor measured 0.4 mm in average dimension, and was the smallest of 7 tumors appearing among 37 mice. × 150

FIGURE 27.—Microscopic carcinoma of ectocervix near external os induced in strain 129/J castrate exposed to a methylcholanthrene-coated thread for 4 weeks, followed by systemic exposure to a 10 percent diethylstilbestrol pellet. Latent period was 15.7 months. Tumor measured 0.3 mm in average dimension and was the smallest of 6 carcinomas appearing among 34 mice. × 150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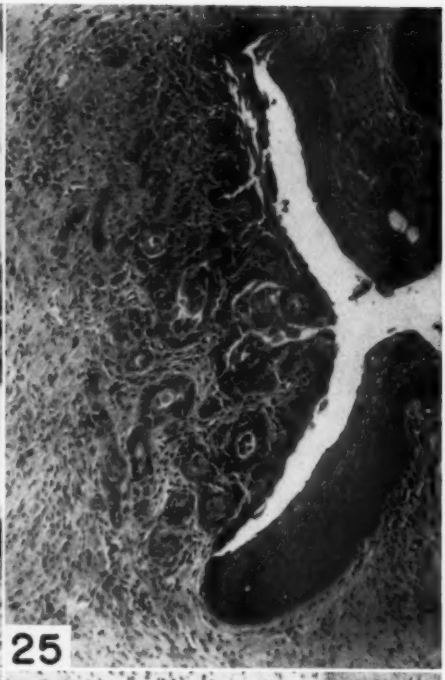
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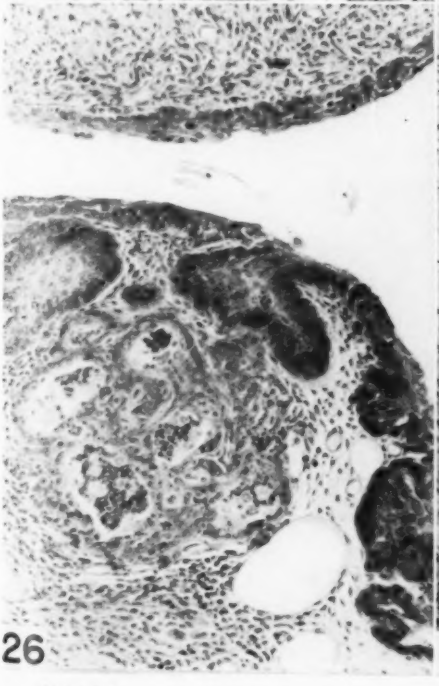
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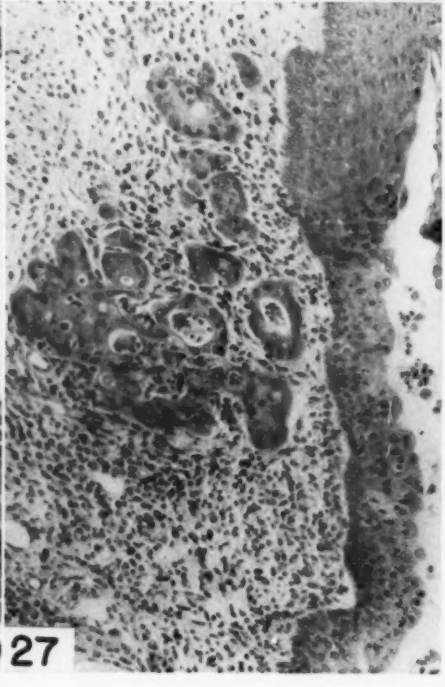
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Mode of Inheritance of Resistance to Rous Sarcoma Virus in Chickens [1]

NELSON F. WATERS *and* B. R. BURMESTER, *Regional Poultry Research Laboratory,*[2] *East Lansing, Michigan*

SUMMARY

This study reports on the mode of inheritance of resistance and susceptibility to intracerebral inoculation with Rous sarcoma virus among inbred White Leghorn chickens. A total of 105 families involving 2,091 chickens was obtained from inbred lines 6, 7, 9, 10, and 15 and from reciprocal-cross families from 6 × 7, 7 × 9, 7 × 15, and 10 × 15. Most dams and sires produced two kinds of progeny: inbred and inbred-cross. Families of chickens from all sires and dams within lines 6 and 10 were all susceptible, while those produced in line 7 were all resistant to Rous sarcoma virus. The families of chickens from different sires and dams within lines 9 and 15 showed a variable response to Rous sarcoma virus. The data presented are in agreement with the theory that susceptibility to intracerebral inoculation with Rous sarcoma virus is dominant to resistance and is dependent for expression upon a single pair of autosomal genes.—J. Nat. Cancer Inst. 27: 655–661, 1961.

BURMESTER *et al.* (*1*) have demonstrated marked differences in the response of inbred lines of chickens when inoculated either subcutaneously or intracerebrally with Rous sarcoma virus (RSV). Waters and Fontes (*2*) showed that differences in mortality due to RSV inoculations occurred in progeny from different sires and dams, both between and within highly inbred lines of White Leghorn chickens. The progeny from both sires and dams within some lines were either completely resistant or completely susceptible in response to inoculation with RSV. Within other lines considerable genetic diversity existed among the progeny from individual sires and dams. In the present study using chickens from the same lines previously reported (*2*), we attempted to demonstrate whether the mode of inheritance for and resistance susceptibility to RSV can be determined.

MATERIAL AND METHODS

All the chickens in this study were White Leghorns and represented 5 unrelated inbred lines. The coefficient of inbreeding for the chickens in

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all lines is now over 95 percent. A brief history of these lines of chickens and the inoculation techniques used are given by Waters and Fontes (*2*). All chickens were inoculated at 1 day of age by the injection of RSV into the right hemisphere of the brain. Chickens that died during the first 7 days after inoculation were excluded from the data, and all experiments were terminated 35 days after the chickens were inoculated.

The RSV preparation was a resuspended centrifuged sediment of an extract of subcutaneous tumors induced by inoculation of preparation CT750, a purified microsome fraction of Rous sarcoma obtained from the National Cancer Institute, Bethesda, Maryland. Comparative assays conducted by intracerebral inoculation of susceptible chicks showed that this RSV preparation was weaker than the original CT750 by about 2 log dilutions. Thus for response results comparable to those reported earlier (*2*), a dose of −3.0 logs instead of −5.0 logs was used for the chickens of the present experiment.

It has been established that mortality in chickens after intracerebral inoculation with RSV is proportional to the dose administered (*3*). Thus, a high concentration of virus preparation will overwhelm the host and cause a high mortality, and conversely a low concentration of virus will show a low mortality. The "optimum" inoculating dose of virus needed to measure resistance and susceptibility must be previously determined by dilution studies of each virus preparation used. Under the conditions of the present study, all chickens that died after inoculation with RSV were considered susceptible, and all those that survived were considered resistant.

Of the 2,091 chickens inoculated, 725 survived until the 35-day experimental holding period. Approximately 200 survivors were autopsied at termination date or soon after. Among this group there was no obviously significant gross pathology and therefore the remaining chickens were destroyed without examination.

RESULTS

Progeny from different sires and dams representing 5 different inbred lines of chickens, together with reciprocal crosses between some of these lines, were inoculated intracerebrally with RSV at 1 day of age. In most instances a sample of progeny from each family studied was produced from individual sires and dams within each of the lines involved. After a sample progeny was produced from each sire and dam, the sires were removed from the pens, and those from different lines were substituted. Samples of inbred-cross progeny were obtained from as many dams as possible. Thus, every sire and most of the dams produced two kinds of progeny: inbred and inbred-cross. The mortality after inoculation with RSV into 1-day-old chickens obtained from both the pure lines and reciprocal crosses between these lines is shown in table 1. Families of chickens were obtained from lines 6 × 7, 7 × 15, 7 × 9, and 10 × 15.

TABLE 1.—Ratios of dead to surviving progeny produced by single sire and dam matings within inbred lines and between crosses of inbred line, after inoculation at 1 day of age with RSV

	Line 6 sire T209J	Line 7 sire T338B		Line 7 sire T338B	Line 6 sire T209J
Line 6 dams	Line 6 progeny	Line 7×6 progeny	Line 7 dams	Line 7 progeny	Line 6×7 progeny
1	20/0*	13/0	9	0/10	12/0
2	9/0	14/0	10	0/15	9/0
3	14/0	9/0	11	0/15	4/0
4	17/0	12/0	12	0/16	7/0
5	16/0	10/0	13	0/16	8/0
6	6/0	7/0	14	0/22	4/0
7	17/0	17/0	15	0/24	5/0
8	16/0	6/0	16	0/10	5/0
Total	115/0	88/0	Total	0/128	54/0

	Line 7 sire U333P	Line 15 sire U719M2		Line 15 sire U719M2	Line 7 sire U333P
Line 7 dams	Line 7 progeny	Line 15×7 progeny	Line 15 dams	Line 15 progeny	Line 7×15 progeny
44	0/11	10/7	57	12/5	1/1
45	0/8	8/8	58	4/0	
46	0/16	10/9	59	4/4	0/20
47	0/1	10/6	60	11/9	
48	0/5	14/2†	61	9/6	
49	0/2	6/7	62	14/6	1/2
50	0/6	9/7	63	14/0	14/0
51	0/3	7/8	64		0/2
52	0/13	7/7	65	17/3	4/3
53	0/2	3/2	66	2/3	0/2
54	0/12	15/6†			
55	0/15	13/10			
56	0/15	6/9			
Total	0/109	118/88	Total	87/36	20/30

	Line 10 sire U509J	Line 15 sire U750B		Line 15 sire U750B	Line 10 sire U509J
Line 10 dams	Line 10 progeny	Line 15×10 progeny	Line 15 dams	Line 15 progeny	Line 10×15 progeny
83	3/0	21/0	94	0/4	12/0
84		10/0	95	0/2	21/0
85		8/0	96		21/0
86		11/0	97		18/0
87	2/0	5/0	98	0/8	17/0
88	2/0	13/0	99	2/3	2/0
89	2/0	3/0	100	0/2	16/0
90		14/0	101	0/15	24/0
91	2/0	6/0	102		16/0
92	5/0		103	12/0	20/0
93	1/0	27/0	104	0/1	14/0
			105	0/2	17/0
Total	17/0	118/0	Total	14/37	198/0

	Line 6 sire U203B	Line 7 sire U338E		Line 7 sire U338E	Line 6 sire U203B
Line 6 dams	Line 6 progeny	Line 7×6 progeny	Line 7 dams	Line 7 progeny	Line 6×7 progeny
17	5/0	2/0	31	0/5	13/0
18	12/0	3/0	32	0/9	20/0
19	13/0	3/0	33	0/2	22/0
20	4/0	11/0	34		13/0
21	14/0	13/0	35	0/8	14/0
22		13/0	36		20/0
23	18/0	6/0	37		18/0
24		12/0	38		19/0
25		14/0	39	0/12	14/0
26		3/0	40		6/0
27	12/0	1/0	41	0/10	25/0
28	14/0	6/0	42	0/7	5/0
29	16/0	15/0	43		14/0
30	4/0	17/0			
Total	112/0	119/0	Total	0/53	203/0

	Line 9 sire U458C	Line 7 sire U373J		Line 7 sire U373J	Line 9 sire U458C
Line 9 dams	Line 9 progeny	Line 7×9 progeny	Line 7 dams	Line 7 progeny	Line 9×7 progeny
67	12/5	10/9	76		16/18
68	10/5	8/7	77	0/8	5/3
69	3/1	4/4	78	0/3	
70	3/15†	9/0	79		7/6
71	2/6†	0/17	80	0/6	0/1
72	5/10†	8/7	81	0/9	17/14
73	13/4	14/13	82	0/1	
74		4/4			
75		6/6			
Total	48/46	63/76	Total	0/27	45/42

*Ratio = dead/survivors. †Divergent ratios.

After inoculation with RSV, all chickens in each family within lines 6 and 10 died before the end of the experimental period, and hence were classified as susceptible. None of the chickens within each family of line 7 died after inoculation, and they were classified as resistant to RSV. The families of chickens from different sires and dams within lines 9 and 15 showed a variable mortality response to inoculation with RSV. Different mating combinations within these two lines produced some families completely resistant in their response and some families either completely or partially susceptible to RSV. The mortality responses occurring in the chickens of the 5 different lines reported are similar to those found previously (*2*) when samples of chickens from these same 5 lines were inoculated with RSV.

Two sets of reciprocal-cross progeny were produced from lines 6 and 7. The mortality responses after inoculation with RSV among the families from both sets of 6 × 7 and 7 × 6 reciprocals were similar. All chickens from lines 6 for both sets of matings were susceptible to RSV; all from line 7 were resistant. Chickens obtained from each of the dams from both sets of reciprocal crosses of lines 6 and 7 were completely susceptible.

Reciprocal inbred-cross chickens produced from lines 10 and 15 were all susceptible and died after inoculation. All families of chickens obtained from line 10 were susceptible. The families of chickens from line 15, used in the reciprocal crosses between lines 10 and 15, were variable in their mortality response to inoculation. The 100 percent mortality occurring among the reciprocal inbred-cross progeny obtained from a cross between lines 6 and 7 and between 10 and 15 suggests that genetic susceptibility to RSV is a dominant trait.

The data obtained from individual families of chickens produced from the reciprocal crosses between lines 7 and 15 and 7 and 9, with few exceptions, strengthen the hypothesis that susceptibility to RSV is not only completely dominant to resistance at the dose level used, but also that this dominant trait is dependent upon a single pair of autosomal genes for expression.

A detailed examination of the mortality within families of chickens, produced from a cross between line-15 sire U719M2 and resistant line-7 dams, shows that 11 of 13 families approached a 1:1 ratio of 89 susceptible and 80 resistant chickens to RSV. These results indicate that sire U719M2 is heterozygous. The two divergent families are from dams 48 and 54. Additional information favoring a single pair of genes to explain the inheritance of resistance and susceptibility to RSV is provided from results obtained from mating the heterozygous sire U719M2 with line-15 dams. When this sire is mated to 9 of the line-15 dams, the evidence provided by the mortality response of their resultant families of chickens indicates the mixed genotypes of these dams. Dams 57, 62, and 65 were all heterozygous and produced progeny in a 3:1 ratio of 43 susceptible and 14 resistant progeny; dams 58 and 63 were dominantly homozygous and produced all susceptible progeny. Dams 59, 60, 61, and 66 were recessive and produced progeny in a 1:1 ratio of 26 susceptible to 22

resistant. Thus, the ratio of susceptible to resistant chickens produced by each of 9 different line-15 dams, when mated with sire U719M2, closely approaches expectation. Likewise, the mortality results obtained among the families of chickens, involving a cross between line-7 recessive sire U333P and these same line-15 dams, is in agreement with the suggested genotypes of these dams.

Mortality data in response to inoculation with RSV are presented for both lines 7 and 9, together with reciprocal matings between chickens from these two lines. The line-9 sire U458C can be considered heterozygous, as demonstrated by the performance of this sire when he is crossed with 5 line-7 resistant dams. The progeny, resulting from this cross of sire U458C with these line-7 dams, closely fits a 1:1 ratio of 45 susceptible and 42 resistant chickens. Considerable variability in mortality from RSV occurred among chickens from the different line-9 families produced by different line-9 dams mated with sire U458C. Dams 67, 68, 69, and 73 produced progeny showing an acceptable fit to a 3:1 ratio of 38 chickens susceptible and 15 resistant. Progeny behavior in 3 of the line-9 families does not fit expectation completely, based on the theory of a single pair of genes. Dams 70 and 72 should produce progeny in a ratio of 3 susceptible to 1 resistant, but actually 8 progeny were susceptible and 25 resistant. However, when these two dams are backcrossed to the recessive line-7 sire U373J, the resultant progeny do show a 1:1 ratio of 17 susceptible and 16 resistant. Dam 71, when mated with a heterozygous line-9 sire, while divergent in ratio of dead to surviving progeny, may be classified as recessive. This assumption is strengthened by the behavior of its progeny, none dead to 17 survivors, when mated to a recessive line-7 sire. All the line-9 dams, except dam 71, when backcrossed with the recessive line-7 male U373J, produced progeny in a ratio of 63 susceptible to 59 resistant, and this ratio is compatible with expectation for a backcross population.

DISCUSSION

Several examples, showing that genetic resistance and susceptibility of host animals to induced disease is dependent on specific and demonstrable gene action, are available for study. Sabin (*4*) demonstrated that PRI albino mice in response to induced 17D-strain yellow-fever virus are completely resistant, whereas Swiss mice are 100 percent susceptible. A cross between the PRI albino mice and Swiss mice suggests that resistance to 17D-strain yellow-fever virus is dependent upon a single autosomal dominant factor for expression. Heston *et al.* (*5*, *6*) presented evidence for gene control which differentiates between resistant and susceptible mice for the propagation and transmission of mammary-tumor agent. Waters *et al.* (*7*) showed that resistance to erythroblastosis (using chickens from the same inbred lines in the present study) involves a relatively simple type of inheritance and is seemingly dependent for

complete expression on only 1 pair of autosomal dominant genes. Possibly none of the experiments cited would have yielded a precise genetic interpretation without known inbred-host material with which to work.

The genetic conclusions of the present study regarding resistance and susceptibility to RSV agree remarkably well with those of Prince (*8*). This investigator, inoculating RSV on the chorio-allantoic membrane of embryos from two different breeds of chickens and their reciprocal crosses, concluded that sensitivity (*susceptibility*) to RSV inoculation was dominant to resistance and that the data were compatible with the hypothesis that this trait was controlled by a single pair of autosomal allelic genes. Further comparison of the genetic results obtained by Prince and those revealed in the present study provides the interesting fact that the routes of inoculation of RSV in both studies were not only different but that they involved pre-embryonic and postembryonic chicken tissue. Likewise, Burmester *et al.* (*1*) demonstrated marked genetic differences in response to RSV when chickens, from some of the same inbred lines used in this study, were inoculated in the wing web. Additional evidence on the genetic resistance of chickens to RSV (*9*) and other transmissible sarcomas is available (*10–12*).

The present studies were projected in an attempt to reveal the mode of inheritance of resistance and susceptibility to intracerebral inoculation of 1-day-old chickens with RSV. The data collected represent a continuation of the previous studies (*2*) which demonstrated marked genetic variation among families of chickens involving 5 unrelated inbred lines. All chickens used in the previous trials (*2*) and in the present study in line 7 were completely resistant; those from lines 6 and 10 were nearly all susceptible, and those from lines 9 and 15 showed considerable heterogenicity.

After measurement of the mortality response to inoculation with RSV of the different lines and families within these lines, the males were exchanged between some of the lines and reciprocal groups of inbred-cross progeny from the same dams, but different sires were obtained. The data presented clearly establish that the chickens tested in the different families of lines 6 and 10 all died before 35 days of age and that they can be considered genetically susceptible in their response to inoculation with RSV. The data likewise establish that all chickens in line 7 were genetically resistant to inoculation with RSV. The variable mortality, resulting from inoculation with RSV, among the families of chickens in both lines 9 and 15 demonstrates that the sires and dams producing these families were of mixed genotypes.

Reciprocal crosses between lines 6 and 7 and between 10 and 15 indicate that susceptibility to RSV is a dominant trait. Reciprocal crosses between lines 7 and 15 and between 7 and 9, with few exceptions, provide 3:1 and 1:1 genetic ratios that do not deviate significantly from expectation in all families of chickens studied, as tested by the chi-square method. Thus, on the basis of the dose level used in this study, it is established that susceptibility in response to intracerebral inoculation

with RSV is dominant to resistance and dependent for expression on a single pair of autosomal genes.

REFERENCES

(1) Burmester, B. R., Fontes, A. K., Waters, N. F., Bryan, W. R., and Groupé, V.: The response of several inbred lines of White Leghorns to inoculation with the virus of Strain RPL 12 visceral lymphomatosis-erythroblastosis and of Rous sarcoma. Poultry Sc. 39: 199–215, 1960.

(2) Waters, N. F., and Fontes, A. K.: Genetic response of inbred lines of chickens to Rous sarcoma virus. J. Nat. Cancer Inst. 25: 351–357, 1960.

(3) Groupé, V., Rauscher, F. J., Levine, A. S., and Bryan, W. R.: The brain of newly hatched chicks as a host-virus system for biological studies on the Rous sarcoma virus (RSV). J. Nat. Cancer Inst. 16: 865–875, 1956.

(4) Sabin, A. B.: Nature of inherited resistance to viruses affecting the nervous system. Proc. Nat. Acad. Sc. 38: 540–546, 1952.

(5) Heston, W. E., Deringer, M. K., and Dunn, T. B.: Further studies on the relationship between the genotype and the mammary tumor agent in mice. J. Nat. Cancer Inst. 16: 1309–1334, 1956.

(6) Heston, W. E., Vlahakis, G., and Deringer, M. K.: Delayed effect of genetic segregation on the transmission of the mammary-tumor agent in mice. J. Nat. Cancer Inst. 24: 721–731, 1960.

(7) Waters, N. F., Burmester, B. R., and Walter, W. G.: Genetics of experimentally induced erythroblastosis in chickens. J. Nat. Cancer Inst. 20: 1245–1256, 1958.

(8) Prince, A. M.: Quantitative studies on Rous sarcoma virus. II. Mechanism of resistance of chick embryos to chorio-allantoic inoculation of Rous sarcoma virus. J. Nat. Cancer Inst. 20: 843–850, 1958.

(9) Greenwood, A. W., Blythe, J. S. S., and Carr, J. G.: Indications of the heritable nature of nonsusceptibility to Rous sarcoma in fowls. Brit. J. Cancer 2: 135–143, 1948.

(10) El Dardiry, A. H., Waters, N. F., and Cottral, G. E.: The response of inbred lines of chickens to lymphoid tumor transplants. Poultry Sc. 31: 523–534, 1952.

(11) Cole, R. K.: Genetic resistance to a transmissible sarcoma in the fowl. Cancer Res. 1: 714–720, 1941.

(12) Stubbs, E. L.: Experiments in resistance to fowl sarcoma, strain 13. Am. J. Vet. Res. 4: 368–373, 1943.

The Hepatocarcinogenicity of a Series of 4′-Alkyl-4-Dimethylaminoazobenzenes [1, 2]

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SUMMARY

The following 4′-alkylated derivatives of dimethylaminoazobenzene (DAB) have been tested for hepatocarcinogenic activity in the rat: 4′-Me(1), 4-Et(16), 4′-*n*-Pr(6), 4′-*iso*-Pr(9), 4′-*n*-Bu(1), 4′-*iso*-Bu(0), 4′-*sec*-Bu(0), 4′-*tert*-Bu(1), and 4′-phenylDAB(0). Parentheses after each indicate its rough quantitative activity. No explanation is apparent for this difference in activity.—J. Nat. Cancer Inst. 27: 663–666, 1961.

ALL THE monomethyl substituted dimethylaminoazobenzenes (DAB) have been tested for hepatocarcinogenic activity (*1*). A wide variation in activity was noted, and the 4′-methyl compound was given a value <1. One might then assume that alkylation in the 4′-position has a tendency to reduce carcinogenicity. However, somewhat later, Sugiura and coworkers (*2*) reported that 4′-ethyl-4-methylaminoazobenzene (MAB) had a high activity. Since it has been demonstrated that the methyl derivatives of both DAB and MAB have essentially the same activity (*3*, *4*), it could be assumed that 4′-ethylDAB would have a high activity. This was confirmed by Miller and coworkers (*5*) who found that 12 of 13 rats fed this compound for 4 months developed tumors. No satisfactory explanation was advanced for the difference in activity of the 4′-methyl and 4′-ethyl compounds (*2*), and it seemed of interest to investigate further the 4′-alkylated derivatives.

MATERIALS AND METHODS

The known 4′-substituted derivatives of DAB were crystallized from ethanol, chromatographed on alumina in benzene solution, and then re-

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crystallized to constant melting point from ethanol (*6–11*). They are listed with reference and melting point: 4′-methyl (*6*), 170–1° C; 4′-ethyl (*7, 8*), 131–2° C; 4′-*iso*propyl (*9*), 105–6° C; 4′-*tert*-butyl (*10*), 142° C; 4′-phenyl (*11*), 208° C. The 4 new compounds were prepared by diazotization of the *p*-alkylaniline and coupling with *N,N*-dimethylaniline in the usual way. They were all purified as stated. Table 1 lists their melting points, analyses, and references to the aniline from which they were prepared.

TABLE 1.—Preparation of new 4′-alkylDAB

4′-Alkyl group	Reference to *p*-alkylaniline	mp ° C	Analyses					
			Calculated (%)			Found (%)		
			C	H	N	C	H	N
n-propyl	(*12*)	100	76. 35	7. 92	15. 72	76. 25	7. 65	15. 92
n-butyl-	(*13*)	67–8	76. 82	8. 24	14. 93	76. 68	8. 30	15. 10
*iso*butyl-	(*14*)	86–7	76. 82	8. 24	14. 93	76. 80	8. 31	15. 19
sec-butyl	(*15*)	55–6	76. 82	8. 24	14. 93	76. 73	8. 08	15. 22

Young male rats of the Sprague-Dawley strain, approximately 8 weeks of age and weighing 150 to 200 g, were distributed as equally as possible by initial body weights into groups of 10 animals each. The diet, feeding, and conditions have been previously described (*16*). Laparotomies were performed at 2-month intervals, and gross and microscopic examinations were carried out on the extirpated livers at the end of the experiment. The microscopic examinations were conducted by Dr. George T. Hoffmann of St. Vincent's Hospital, New York City. His report indicated that the tumors were of the same type reported in our previous work (*16*).

RESULTS

Table 2 shows the tumor incidence when rats were fed the dye at the 0.06 percent level. The indications are that the 4′-ethyl and 4′-*iso*propyl compounds are quite active, but their toxicity precluded satisfactory evaluation at this level of feeding.

TABLE 2.—Tumor incidences (0.06% level)*

Compound code	4 Months	6 Months	X Months
DAB	7/10	9/10	
4′-MeDAB	0/10	0/10	10–0/10
4′-EtDAB	7/7 Toxic		
4′-*n*-PrDAB	5/9	7/9	
4′-*iso*PrDAB	3/3 Toxic		
4′-*n*-BuDAB	0/10	0/9	12–3/7
4′-*iso*BuDAB	0/8	0/8	12–0/8
4′-*sec*BuDAB	0/8	0/8	12–0/8
4′-*tert*BuDAB	0/10	0/9	12–3/9
4′-phenylDAB	0/10	0/10	

* Number of rats with hepatic tumors / Number of rats in experiment

The 4′-*n*-propylDAB showed appreciable activity which was somewhat less than that of DAB. Both 4′-*n*-butyl and 4′-*tert*-butylDAB showed slight activity. The other compounds produced no tumors in a 12-month period.

Table 3 shows a comparison of DAB with the 4′-ethyl and 4′-*iso*propyl compounds at the 0.03 percent feeding level. The Miller formula (*3*),

$$\text{Relative activity} = \frac{6 \times \text{months fed} \times \text{percent tumors of test compound}}{\text{months fed} \times \text{percent tumors of DAB}},$$

was applied to these compounds and rough quantitative values were assigned. We used weight percent in our experiments; the values would be slightly higher had we used mole percent as done by previous workers.

TABLE 3.—Tumor incidences (0.03% level)*

Compound code	4 Months	6 Months	Rough quantitative activity
DAB	0/10	5/10	6
4′-EtDAB	8/10	10/10	16
4′-*iso*PrDAB	1/10	7/9	9

*$\frac{\text{Number of rats with hepatic tumors}}{\text{Number of rats in experiment}}$.

Our results certainly show that alkylation in the para position (4′-position) does not necessarily reduce carcinogenic activity. Unfortunately no pattern emerges from our data to indicate any mechanism for the carcinogenesis.

REFERENCES

(*1*) MILLER, J. A., and MILLER, E. C.: The carcinogenic aminoazo dyes. Advances Cancer Res. 1: 339–396, 1953.

(*2*) SUGIURA, K., CROSSLEY, M. L., and KENSLER, C. J.: The carcinogenicity of ethyl derivatives of *p*-aminoazobenzene. J. Nat. Cancer Inst. 15: 67–72, 1954.

(*3*) MILLER, J. A., and MILLER, E. C.: The carcinogenicity of certain derivatives of *p*-dimethylaminoazobenzene in the rat. J. Exper. Med. 87: 139–156, 1948.

(*4*) SUGIURA, K.: The carcinogenicity of certain compounds related to *p*-dimethylaminoazobenzene. Cancer Res. 8: 141–144, 1948.

(*5*) MILLER, J. A., MILLER, E. C., and FINGER, G. C.: Further studies on the carcinogenicity of dyes related to 4-dimethylaminoazobenzene. The requirement for an unsubstituted 2-position. Cancer Res. 17: 387, 1957.

(*6*) MOHLAN, R.: Ueber eine Darstellungsmethode des Helianthins. Ber. 17: 1490–1493, 1884.

(*7*) SAWICKI, E., and RAY, F. E.: The relation of physical properties of carcinogens to their activity. I. The basicity of alkylated 4-aminoazobenzene dyes. J. Org. Chem. 19: 1686–1691, 1954.

(*8*) SAWICKI, E.: Physical properties of aminoazobenzene dyes. V. The C_4/A_4 ratio. J. Org. Chem. 22: 621–625, 1957.

(*9*) SAWICKI, E., and GERBER, D.: The physical properties of aminoazobenzene dyes. II. Further studies of the basicity. J. Org. Chem. 21: 410–412, 1956.

(*10*) CAMPBELL, T. W., YOUNG, D. A., and ROGERS, M. T.: The electric moments of some azo dyes. J. Am. Chem. Soc. 73: 5789–5791, 1951.

(*11*) VORLANDER, D.: Die Lehre von den innermolekularen Gegensätzen und die Lenkung der Substituenten im Benzol (II). Ber. 58: 1893–1914, 1925.

(*12*) LOUIS, E.: Ueber einige aromatische Amine. Ber. 16: 105–116, 1883.

(*13*) READ, R. R., and MULLIN, D. B.: Derivatives of normal butylbenzene. J. Am. Chem. Soc. 50: 1763–1765, 1928.

(*14*) HICKINBOTTOM, W. J., and PRESTON, G. H.: The rearrangement of alkylanilines. Part III. The formation of *p*-amino-*iso*-butylbenzene and of *p*-amino-*tert*-butylbenzene from *iso*-butylaniline. J. Chem. 1566–1571, 1930.

(*15*) GLATTFELD, J. W. E., and WERTHEIM, E.: The preparation of optically-active hydrazines. II. The preparation of *dl*-*p*-secondary-butylphenylhydrazine. The resolution of *dl*-*p*-secondary-butyl-aniline. J. Am. Chem. Soc. 43: 2682–2687, 1921.

(*16*) BROWN, E. V., *et al.*: Carcinogenic activity of some heterocyclic analogs of *p*-dimethylaminoazobenzene; effects of methyl groups in pyridine ring. Cancer Res. 14: 715–717, 1954.

β-Glucuronidase Activities of Cultured Cells Derived From C3H Mouse Liver [1]

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SUMMARY

Three related strains of cells derived from infant C3H mouse liver and maintained in culture for over 12 years had very high β-glucuronidase levels. On a nitrogen basis, the activities were 60 to 100 times the average value for adult C3H liver and 20 to 30 times the value observed in the livers of 2-day-old mice. The high activities persisted in tumors obtained by implanting the cultured cells into C3H mice. Regenerating C3H liver and a variety of spontaneous and transplanted C3H hepatomas were assayed for β-glucuronidase activity; in none of these was the level significantly higher than that in infant liver. One ascites hepatoma was adapted to and carried in tissue culture for 2 years without any increase in activity. Different strains of cultured C3H fibroblasts exhibited widely differing β-glucuronidase activities. A few were as active as the liver cultures; but others, although exposed to the culture environment for an equally long period, had much less activity. β-Glucuronidase activity was localized chiefly within the particulate fraction of sucrose homogenates of the cultured liver cells. The enzyme exhibited a high degree of latency and a *p*H optimum at 4.4. These properties suggested that the activity was associated with lysosome-like particles in the cultured cells. Compared to adult C3H liver, acid-phosphatase activity was increased threefold in the cultured cells, while ribonuclease activity was unchanged. Uricase activity was not detectable in the liver cultures.—J. Nat. Cancer Inst. 27: 667-678, 1961.

TISSUES OF C3H mice (Andervont, Heston, and Bittner sublines) exhibit lower levels of β-glucuronidase activity than do those of most other inbred mouse strains (*1–5*). The differences are particularly marked in the liver, where glucuronidase activities in C3H mice are only 10 to 20 percent of those found in mice of the usual "high-activity" strains (*1*, *2*). Law, Morrow, and Greenspan first showed that the enzyme level in this organ is under genetic control and that the C3H strain is apparently homozygous in a recessive allele determining low activity (*6*).

In searching for a mammalian system that might be suitable for transformation studies such as those with bacteria, we assayed the β-glucuroni-

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dase activity in strains of cultured cells originally derived from C3H mouse liver (*7–9*). Paradoxically, these cells and tumors derived from them have consistently shown β-glucuronidase levels many times higher than even the livers of high-activity mice. This observation led to the investigation of other types of cultured C3H cells and to the finding, reported elsewhere (*10*), of marked differences in β-glucuronidase activity among various clones of cultured fibroblasts. The results of our studies on the liver cultures themselves are reported in this paper.

MATERIALS AND METHODS

Cultured cell lines.—Three strains of cells derived from C3H mouse liver were studied. The derivation of these strains, presented in detail elsewhere (*7–9*), may be briefly summarized. NCTC strain 721 was established in 1948 and arose from an explant of parenchymatous liver tissue from a 2-day-old mouse of the Heston substrain. NCTC clone 1469 represents a clone established in 1952 from a single cell of strain 721. NCTC strain 1795 was established in 1953 by explantation of a tumor produced by injection of clone 1469 cells into a C3H mouse. Cells of strain 1795 produce tumors in C3H mice with an incidence of over 50 percent. Those of clone 1469 rarely produce tumors, and strain 721 cells have never given rise to tumors. Strain L fibroblasts of clone 929 and strain 2071 (*11*), also of C3H origin, were assayed.

Inbred mice.—Unless otherwise stated, C3H mice were all of the Heston substrain (C3H/HeN) and carried the mammary-tumor agent. The animals used for the biochemical studies belonged to the 42d to 50th inbred generations of this substrain. Strain A mice of the A/LN substrain, inbred for 109 generations, were also studied.

Transplantable hepatomas.—All hepatomas arose and have been carried in C3H mice of the Heston or Andervont substrains. Hepatoma 98/15 developed spontaneously (*12*). Solid hepatoma 129 arose in a mouse that had received carbon tetrachloride (*13*). Ascites hepatomas 129(P) and 129(F) were derived from the solid tumor 129 (*14*), and ascites tumor 134 from another transplantable hepatoma obtained originally in a carbon-tetrachloride-treated mouse (*13*).

Spontaneous hepatomas.—Six tumors were assayed. All arose in C3H mice that were free of the mammary-tumor agent and belonged to a substrain designated C3Hf/He.[3]

Tissue culture.—Cells were grown in T-60 flasks (*7*), modified roller tubes (*15*), or agitated fluid suspensions as previously described (*16*). The following culture media were used (*see* tables 1 and 3): (*a*) a mixture of 30 percent horse serum, 20 percent chick-embryo extract, and 40 percent Earle's saline, designated H.S.-E.E., (*b*) a completely defined protein-free medium, NCTC 109 (*11, 17*), and (*c*) NCTC 109 supplemented with 10 or 20 percent horse serum, designated NCTC 109-H.S.

[3] These animals were kindly supplied by Dr. W. E. Heston, of the National Cancer Institute.

Preparation of homogenates.—Cultured cells were suspended in the culture medium by gentle scraping or agitation, removed from the flasks, chilled rapidly to 0° C, and sedimented by centrifugation for 5 minutes at 1000 rpm (220 × *g*) in rotor #269 of the International PR2 centrifuge. The packed cells were resuspended in ice-cold 0.25 M sucrose and immediately resedimented in calibrated tubes. The clear wash fluid was removed and the pellet (averaging about 0.05 ml per T-60 flask) was resuspended and homogenized (*18*) in 10 to 20 volumes of distilled water or 0.25 M sucrose. Ascites tumor cells were removed from the animals on the 7th day after transplantation and similarly treated. Solid tissues were homogenized without prior treatment. The particulate and "soluble" fractions of homogenates prepared in 0.25 M sucrose were separated by centrifugation for 1 hour at 35,000 rpm (135,000 × *g*) in the SW-39 rotor of the Spinco Model L ultracentrifuge.

Enzyme assay.—β-Glucuronidase is associated chiefly with particulate structures in homogenates of mouse and rat livers when such homogenates are prepared in saline or isotonic sucrose (*19*, *20*). Much of the bound enzyme appears to have only limited activity toward added substrates; however, full activity may be developed by various procedures that disrupt the particles and render the enzyme soluble (*21*, *22*). In the present study, both sucrose and water homogenates were routinely preincubated for 3 hours at 37° C at a dilution of 1:4 in 0.01 M acetate buffer, *p*H 5.0, when complete activation of the enzyme was desired (*21*). The activity of the preincubated material was then determined at *p*H 5.0 by a modification (*10*) of the method of Talalay, Fishman, and Huggins (*23*), with phenolphthalein glucuronide (Sigma Chemical Co., St. Louis, Mo.) as the substrate. Determinations of the proportions of latent and active enzyme in sucrose homogenates and in fractions prepared therefrom were carried out essentially according to the method of de Duve *et al.* (*22*). A unit of β-glucuronidase activity is defined as that activity which results in the release of 1 μg of phenolphthalein in 60 minutes at 37° C. The results embodied in tables 1 to 3 have been expressed as units of activity per mg total tissue nitrogen.

Acid phosphatase (*21*) and ribonuclease (*24*) activities were assayed at *p*H 5.0 in preincubated homogenates by the procedures described in the appropriate references. Uricase activity was measured spectrophotometrically at *p*H 9.0 (*25*).

Total nitrogen was determined by nesslerization of acid digests (*26*).

RESULTS

It is important to establish, as far as possible, whether the liver explant from which the cultured cell line was derived in 1948 (*7*) was indeed taken from a mouse belonging to a low-activity strain. Enzymatic assays were not carried out at the time. Fortunately, a number of individuals of the same colony of C3H mice (Heston substrain, obtained from Andervont

in 1941) were tested during 1949 and uniformly showed low liver activity (*1*, *2*). Since the Andervont-Heston substrain had at that time already been intensively inbred for 19 years, it seems almost certain that the cultured cells were derived from a mouse that was homozygous for the recessive allele that determines low activity.

Over the past 3 years, the β-glucuronidase levels in the livers of adult mice of this substrain have averaged 10.4 units per mg nitrogen (table 1). Levels of 80 to 85 units per mg nitrogen were observed in the livers of mice belonging to the high-activity strains A and C (*cf. 1*, *2*).

In striking contrast to these figures were the β-glucuronidase activities observed in the various lines of cultured liver cells (table 1). Activities ranged from 60 to 100 times the average value for adult C3H liver. Since the ratio of protein to deoxyribonucleic acid is essentially the same in the cultures as it is in the intact liver (*27*), the data in table 1 undoubtedly reflect significantly higher *average* levels of activity per cell in the cultured material. The distribution of activity among the morphological cell variants that have been observed in the liver cultures (*9*) is not yet known. The enzyme levels in cells cultured in NCTC 109 with added horse serum were in several instances considerably higher than in the corresponding cells maintained on the horse serum-embryo extract medium.

TABLE 1.—β-Glucuronidase activities in strains of cultured liver cells derived from a C3H mouse and in the livers of adult C3H mice

Culture strain	β-Glucuronidase activity * (units/mg total N)	Culture medium	Remarks
721	655, 675	H.S.-E.E.	Mixed culture from liver of 2-day-old C3H mouse
3177	1220	NCTC 109-H.S.	Strain 721 on different medium
1469	900, 970, 1040	H.S.-E.E.	Clone from strain 721
3080	625, 785	H.S.-E.E.	Clone 1469 in suspension culture
3178	1515, 1980	NCTC 109-H.S.	Clone 1469 on different medium
1795	745, 785	H.S.-E.E.	Mixed culture from tumor produced by clone 1469 in mouse
3179	780	NCTC 109-H.S.	Strain 1795 on different medium
Adult liver	10		Range 7.5 to 13 units per mg nitrogen; 13 animals of both sexes tested

* Each value represents the analysis of an individual sample of cultured cells.

Enzyme levels comparable to these observed in the cultured cells were not seen in infant and regenerating C3H mouse liver or in a variety of hepatomas of C3H origin (table 2). In confirmation of previous studies (*20*), we found that glucuronidase activities in the livers of infant C3H mice were several times greater than those in adult liver. It remains to be determined whether this elevated activity is distributed throughout all of the cells of the infant liver or is confined to a special

TABLE 2.—β-Glucuronidase activities in infant and regenerating C3H livers, primary and transplanted C3H hepatomas, and in tumors derived from implantation of cultured C3H liver cells

Tissue	Number of samples analyzed	β-Glucuronidase activity (units/mg total N)		Remarks
		Average	Range	
Liver, infant mice	2	30. 0	29. 0, 31. 5	Three livers pooled for each sample; mice 2 to 3 days of age
Liver, regenerating	1	6. 1	—	Two days after partial hepatectomy; 2 livers pooled
	2	12. 7	10. 2, 13. 2	Four days after partial hepatectomy
Hepatomas, primary	6	7. 1	4. 6–12. 1	Arose in agent-free mice, both sexes
Hepatomas, transplanted				
Line 98/15	3	36. 2	29. 8–44. 4	Solid tumors
Line 129	3	34. 0	29. 1–43. 2	Solid tumors
Line 129(P)	2	22. 7	22. 4, 23. 0	Ascites tumors
Line 129(F)	2	6. 7	6. 2, 7. 2	Ascites tumors
Line 134	2	19. 0	16. 5, 21. 4	Ascites tumors
Tumors derived from cultured liver cells of strain 1469				
First generation	1	1415	—	Three months after original implantation of cells
Second generation	4	930	885–985	Eleven months after original implantation of cells; 2 months after transplantation of first-generation tumor to new hosts

cell type. This question is clearly of central importance in evaluating the high levels in the cultured cells (*see* Discussion).

Several tumors derived from the implantation of clone 1469 cultures into C3H mice were analyzed (table 2). The single first-generation tumor was assayed 3 months after intramuscular implantation of the culture. The 4 second-generation tumors were transplants of a tumor derived from another culture. All tumors were between 1.5 and 2 cm in diameter when assayed. High levels of β-glucuronidase activity persisted in the tumors in spite of: (*a*) exposure of the cells to the internal milieu of the animals for a prolonged period, (*b*) occurrence of multiple mitotic cycles during this time, and (*c*) dilution of the actively proliferating cells with stromal elements contributed by the hosts. Thus, it appears that the high enzyme levels are a heritable characteristic of the cultured cells and are not, or at least are no longer, contingent upon continued exposure of the cells to the culture environment.

Conversely, prolonged maintenance in culture has not invariably evoked the development of high β-glucuronidase activities in cells of C3H origin

(table 3). Ascites hepatoma 129(P), for example, has exhibited a consistently low activity for over 2 years in culture, though during this period the growth rate has increased greatly as the cells progressively adapted to their new environment. Clone 929 of strain L fibroblasts showed comparatively low activity after many years in culture. There are marked and persistent differences in activity between certain clones of fibroblasts that presumably all originated from a single connective-tissue cell [(*10*) and table 3]. Apparently there was no consistent relationship between the levels of enzyme activity and the various culture media used in the experience summarized in table 3.

TABLE 3.—β-Glucuronidase activities in cultured cell strains derived from C3H mice

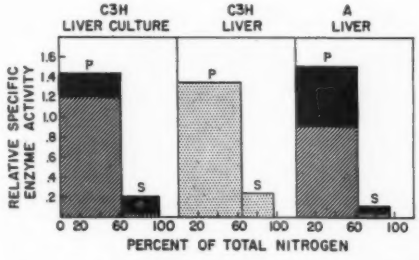
Culture strains	β-Glucuronidase activity (units/mg total N)	Culture medium	Remarks
Fibroblasts (strain L)			
929	73	H.S.-E.E.	Clone, in culture 21 years
2071	210, 210	NCTC 109	Substrain of clone 929 on synthetic medium for 5 years
Fibroblasts*			
1742	60	H.S.-E.E.	Clone, in culture 10 years
2472	43	NCTC 109-H.S.	Subclone from clone 1742
1745	1230	H.S.-E.E.	Separate strains of a clone obtained from a sarcoma produced by clone 1742 cells 9 years ago
2050	1130	H.S.-E.E.	
3069	1110	NCTC 109-H.S.	Substrain of clone 2050
Ascites hepatoma 129(P)			
3225	66, 32	NCTC 109-H.S.	In culture 1 week
	54	" "	" " 1 month
	48	" "	" " 11 months
	52, 49	" "	" " 17 months
	48	" "	" " 29 months

* Data taken from (*10*).

Some properties of the β-glucuronidase enzyme in homogenates of the cultured liver cells have been studied. The *p*H activity curve was compared with the corresponding curves for the enzyme(s) in homogenates of C3H (low-activity) and A (high-activity) mouse livers. Each showed an optimum at *p*H 4.4 to 4.5. The curve for C3H liver showed an additional optimum at *p*H 5.2, as previously reported for homogenates (*2*) but not for partially purified extracts (*28*) of this tissue. Extraneous proteins, nucleic acid, and various low molecular-weight materials are known to influence the effect on β-glucuronidase activity of variations in *p*H (*4*, *5*). In this instance the *p*H activity curves were not significantly altered in shape or position by the addition of 0.02 percent bovine serum

albumin (The Armour Laboratories, Chicago, Ill., 3 times recrystallized) to the assay mixture (*29*) in an effort to minimize the differences in total protein concentration that occurred in comparing homogenates of widely differing activities. Evidence has been presented that the marked variation in liver β-glucuronidase levels among inbred strains of mice is based on quantitative rather than qualitative differences in the enzyme protein (*28*). It will be interesting to determine through the study of more highly purified enzyme preparations whether this is also true with respect to the cultured cells.

The distribution of enzyme activity between the particulate and soluble fractions of sucrose homogenates is illustrated in text-figure 1. The data have been plotted in such a way (*22*) that the area of each bar is proportional to the percentage of the homogenate activity contained in each fraction. Over 85 percent of the activity in liver-culture homogenates was



TEXT-FIGURE 1.—Distribution of β-glucuronidase activity and total nitrogen between particulate (P) and soluble (S) fractions of homogenates of C3H liver culture (strain 1469), adult C3H liver, and strain A liver. Abcissa indicates percentage of total nitrogen of homogenates found in each fraction. Over-all heights of bars are proportional to the specific activities of the fractions (units of activity/mg nitrogen) relative to those of the whole homogenates. Proportions of active and latent enzyme activity are indicated by *solid* and *shaded* areas. This determination was not made for fractions from C3H liver (*see* text).

recovered in the particulate fraction. Similar distributions were obtained for the livers of strain C3H and A mice. In the liver cultures and strain A livers, the proportions of latent and active enzyme were determined for each fraction (*22*); these are indicated by shading within the bars. The enzyme within the particulate fraction of the cultured cells showed a high degree of latency, greater than that observed for the same fraction of strain A mouse liver. The low activity in C3H liver precluded an accurate determination of the proportion of active enzyme. It has been reported, however, that the enzyme in this tissue also shows a higher degree of latency than is found in high-activity livers (*20*).

Extensive studies [summarized in (*30*)] have led to the concept that β-glucuronidase is one of a number of hydrolytic enzymes largely associated with a special class of cytoplasmic particles, the lysosomes, in liver and several other mammalian tissues. Acid *p*H optima and limited reactivities toward their respective substrates in intact preparations are

properties common to the lysosomal enzymes. Paigen has reported that the levels of other lysosomal enzymes are not altered in C3H liver (*20*), and we have confirmed this finding with respect to ribonuclease and acid phosphatase activities. In table 4, the levels of these two enzyme activities in adult C3H liver are compared with those observed in the cultured liver cells. On a nitrogen basis, acid phosphatase was increased more than threefold in the cultured cells, while ribonuclease activity was essentially the same as in the liver. In this instance, the β-glucuronidase level in the cultured cells was about 70 times that observed in the liver.

Uricase activity, which may be associated with still another type of particle within the liver cytoplasm (*31*, *32*), was apparently completely lacking in the cultured cells (table 4). This enzyme also could not be detected in any of the transplantable C3H hepatomas studied. It was slightly reduced in the primary hepatomas and in regenerating liver and was quite low in the livers of infant mice.

TABLE 4.—Comparison of acid phosphatase, acid ribonuclease, uricase, and β-glucuronidase activities in cultured C3H liver cells and adult C3H liver. (Values are for 0.1 ml of packed cultured cells and 0.1 g of liver.)

		Acid phosphatase*		Acid ribonuclease†		Uricase‡		β-Glucuronidase§	
	Total nitrogen (N) mg	Total	Per mg N	Total	Per mg N	Total	Per mg N	Total	Per mg N
Cultured liver cells (strain 3080)	1. 16	275	236	30. 6	26. 4	0	0	725	625
C3H liver	3. 92	252	64. 3	97. 6	22. 4	—	—	33. 5	8. 5
	3. 61	—	—	—	—	10. 3	2. 85	—	—
Cultured cells / Liver			3. 7		1. 2		0		73

*Micrograms phosphorus released in 60 minutes at 37° from β-glycerophosphate, pH 5.0 (*21*).
†Acid-soluble material absorbing at 260 mμ released in 60 minutes at 37° from ribonucleic acid, pH 5.0 (*24*).
‡Decrease in optical density (290 mμ) of uric acid, pH 9.0, in 10 minutes at 23° (*25*).
§Units. (Defined under Materials and Methods.)

DISCUSSION

The β-glucuronidase levels observed in the liver-cell cultures are exceeded in only a few of the many mammalian tissues studied (*4*, *5*). These high activities have been maintained by the cultured cells for more than 2 years; they have also persisted in cells removed from the culture environment and exposed, as tumors, to the internal milieu of intact C3H mice for many months. There was no correlation between the β-glucuronidase levels of the various cell lines and their tumor-producing capacities. Furthermore, while it is not known whether new cultures of C3H liver will regularly acquire high levels of this enzyme, it can at least be stated that such greatly elevated activities are not essential for the active proliferation of all types of C3H cells in tissue culture (table 3).

Our results are fully consistent with previous studies which showed that: 1) β-glucuronidase activities were not elevated in induced rat hepatomas (*33*), and 2) there was no strong correlation between β-glucuronidase levels and rates of cellular proliferation in liver and other normal tissues (*4*, *34*).

The relationship between the cultured liver cells and those of the intact liver is obscure. Tumors produced by the injection of strain 1469 and 1795 cells into mice resemble hepatomas morphologically (*9*). This is probably the strongest indication that the cultured cells were originally derived from parenchymatous liver cells or their precursors in the infant organ. The cultures exhibit some biochemical properties that are characteristic of adult liver tissue, *e.g.*, the capacity for storage and reutilization of glycogen (*35*, *36*) and high arginase and rhodanese activities (*37*). They are deficient, however, in catalase (*38*) and uricase activities. β-Glucuronidase is the first known example of an enzyme activity that is much greater in the cultured cells than in the intact liver.

Recently, evidence was presented that the majority of cells in short-term cultures of infant rat liver were derived from a cell type other than the parenchymal cell (*39*). The possibility must be considered in the present instance that the culture situation may have selectively favored the proliferation of a numerically small group of very high activity cells, parenchymal or otherwise, that were within the original liver tissue. Histochemical investigations of the livers of high-activity animals have failed to demonstrate any such exceptionally active cells (*40–42*). Similarly, our own preliminary applications of two different histochemical methods (*40*, *41*) to C3H liver have provided no indication that the activity in the adult organ was concentrated in a few cells. A definitive answer, however, must await the application to infant liver of techniques with the proved capacity of demonstrating occasional high-activity cells in predominantly low-activity surroundings.

Since we lack a precise definition of the cell of origin, it is impossible to state whether some alteration in the genetic apparatus of the cultured cells underlies their high β-glucuronidase activities. It must be stressed that the culture line was derived from infant liver, which is poorly understood with respect to specific cellular attributes and the potentialities of the cells for development when they are removed from their usual environment. Clearly, interpretation of our data in cytogenetic terms would be facilitated by, and indeed may actually depend upon, observations of freshly isolated C3H liver cells during their development in tissue culture. Such experiments are now being undertaken.

The high degree of latency and the predominantly particulate localization of β-glucuronidase activity in the cultured liver cells suggest that the enzyme was associated there with lysosome-like particles, just as it is in intact C3H liver (*20*). The activities of two other lysosomal enzymes, acid phosphatase and ribonuclease, were not elevated in the cultured cells to the same extent that β-glucuronidase activity was (table 4). A more critical study of the intracellular distribution of these

enzymes in the cultured cells is required to determine whether they are in fact all localized within the same subcellular structures. Meanwhile, there seems to be no *a priori* reason for rejecting the possibility of independent variation in the levels of individual enzymes within the lysosomal particles, either on a genetic basis or as an adaptative response of the cells to their culture environment. It will be of interest to assay the strains of cultured cells listed in tables 1 and 3 for the various enzymes commonly associated with lysosomes in mammalian tissues (*22*, *23*). Such a study may reveal other biochemical differences between the cell strains in addition to aiding in the interpretation of the β-glucuronidase findings.

The recognition of lysosomes as a discrete class of cytoplasmic particles and the hypothesis that they may be concerned with localized phenomena of intracellular digestion (*22*, *30*) have provided a possible insight into the poorly understood role of mammalian β-glucuronidase. Straus has proposed the designation "phagosome" for a type of cytoplasmic vacuole involved in the ingestion and sequestering of foreign materials by mammalian cells (*43*). He has clearly shown that in rat kidney these particles exhibit many of the properties of liver lysosomes, including a concentration of β-glucuronidase activity (*44*). It is therefore interesting that the cultured liver cells commonly showed marked pinocytotic activity and also rapidly ingested polystyrene latex spheres (0.36 μ in diameter) when these were added to the incubation medium. The possible relationship between this functional capacity of the cultured cells and their high β-glucuronidase activity appears worthy of investigation.

REFERENCES

(*1*) Morrow, A. G., Greenspan, E. M., and Carroll, D. M.: Liver-glucuronidase activity of inbred mouse strains. J. Nat. Cancer Inst. 10: 657–661, 1949.

(*2*) ———: Comparative studies of liver glucuronidase activity in inbred mice. J. Nat. Cancer Inst. 10: 1199–1203, 1950.

(*3*) Morrow, A. G., Carroll, D. M., and Greenspan, E. M.: A sex difference in the kidney glucuronidase activity of inbred mice. J. Nat. Cancer Inst. 11: 663–669, 1951.

(*4*) Fishman, W. H.: Beta-glucuronidase. Advances Enzymol. 16: 361–409, 1955.

(*5*) Levvy, G. A., and Marsh, C. A.: Preparations and properties of β-glucuronidase. Advances Carbohydrate Chem. 14: 381–427, 1959.

(*6*) Law, L. W., Morrow, A. G., and Greenspan, E. M.: Inheritance of low liver glucuronidase activity in the mouse. J. Nat. Cancer Inst. 12: 909–916, 1952.

(*7*) Evans, V. J., Earle, W. R., Wilson, E. P., Waltz, H. K., and Mackey, C. J.: The growth in vitro of massive cultures of liver cells. J. Nat. Cancer Inst. 12: 1245–1265, 1952.

(*8*) Hobbs, G. L., Sanford, K. K., Evans, V. J., and Earle, W. R.: Establishment of a clone of mouse liver cells from a single isolated cell. J. Nat. Cancer Inst. 18: 701–707, 1957.

(*9*) Evans, V. J., Hawkins, N. M., Westfall, B. B., and Earle, W. R.: Studies on culture lines derived from mouse liver parenchymatous cells grown in long-term tissue culture. Cancer Res. 18: 261–266, 1958.

(*10*) SANFORD, K. K., WESTFALL, B. B., CHU, E. H. Y., KUFF, E. L., COVALESKY, A. B., DUPREE, L. T., HOBBS, G. L., and EARLE, W. R.: Alterations in morphology, arginase, and β-glucuronidase within a clone of mouse tumor cells *in vitro*. J. Nat. Cancer Inst. 26: 1193–1219, 1961.

(*11*) EVANS, V. J., BRYANT, J. C., MCQUILKIN, W. T., FIORAMONTI, M. C., SANFORD, K. K., WESTFALL, B. B., and EARLE, W. R.: Studies of nutrient media for tissue cells *in vitro*. II. An improved protein-free chemically defined medium for long-term cultivation of strain L-929 cells. Cancer Res. 16: 87–94, 1956.

(*12*) EDWARDS, J. E., DALTON, A. J., and ANDERVONT, H. B.: Pathology of a transplantable spontaneous hepatoma in C3H mouse. J. Nat. Cancer Inst. 2: 555–563, 1942.

(*13*) ANDERVONT, H. B., and DUNN, T. B.: Transplantation of hepatomas in mice. J. Nat. Cancer Inst. (Supp.) 15: 1513–1524, 1955.

(*14*) SATO, H., BELKIN, M., and ESSNER, E.: Experiments on an ascites hepatoma. III. The conversion of mouse hepatomas into the ascites form. J. Nat. Cancer Inst. 17: 1–21, 1956.

(*15*) EARLE, W. R., SCHILLING, E. L., BRYANT, J. C., and EVANS, V. J.: The growth of pure strain L cells in fluid-suspension cultures. J. Nat. Cancer Inst. 14: 1159–1171, 1954.

(*16*) BRYANT, J. C., SCHILLING, E. L., and EARLE, W. R.: Massive fluid-suspension cultures of certain mamalian tissue cells. II. Glucose utilization and cell proliferation. J. Nat. Cancer Inst. 21: 349–364, 1958.

(*17*) EVANS, V. J., BRYANT, J. C., FIORAMONTI, M. C., MCQUILKIN, W. T., SANFORD, K. K., and EARLE, W. R.: Studies of nutrient media for tissue cells in vitro. I. A protein-free chemically defined medium for cultivation of strain L cells. Cancer Res. 16: 77–86, 1956.

(*18*) SCHNEIDER, W. C., and HOGEBOOM, G. H.: Intracellular distribution of enzymes. IX. Certain purine-metabolizing enzymes. J. Biol. Chem. 195: 161–166, 1952.

(*19*) WALKER, P. G.: The preparation and properties of β-glucuronidase 3. Fractionation and activity of homogenates in isotonic media. Biochem. J. 51: 223–232, 1952.

(*20*) PAIGEN, K.: Genetic influences on enzyme localization. J. Histochem. & Cytochem. 7: 248–249, 1959.

(*21*) GIANETTO, R., and DE DUVE, C.: Tissue fractionation studies 4. Comparative study of the binding of acid phosphatase, β-glucuronidase and cathepsin by rat liver particles. Biochem. J. 59: 433–438, 1955.

(*22*) DE DUVE, C., PRESSMAN, B. C., GIANETTO, R. WATTIAUX, R., and APPELMANS, F.: Tissue fractionation studies 6. Intracellular distribution patterns of enzymes in rat-liver tissue. Biochem. J. 60: 604–617, 1955.

(*23*) TALALAY, P., FISHMAN, W. H., and HUGGINS, C.: Chromogenic substrates II. Phenolphthalein glucuronic acid as substrate for the assay of glucuronidase activity. J. Biol. Chem. 166: 757–772, 1946.

(*24*) SCHNEIDER, W. C., and HOGEBOOM, G. H.: Intracellular distribution of enzymes X. Desoxyribonuclease and ribonuclease. J. Biol. Chem. 198: 155–163, 1952.

(*25*) KUFF, E. L., HOGEBOOM, G. H., and DALTON, A. J.: Centrifugal, biochemical, and electron microscopic analysis of cytoplasmic particulates in liver homogenates. J. Biophys. & Biochem. Cytol. 2: 33–54, 1956.

(*26*) UMBREIT, W. W., BURRIS, R. H., and STAUFFER, J. F.: Manometric Techniques and Tissue Metabolism. Minneapolis, Burgess Publishing Co., 1949, p. 191.

(*27*) WESTFALL, B. B.: Personal communication.

(*28*) SIE, H. G., and FISHMAN, W. H.: A comparative study of certain properties of liver β-glucuronidase of inbred mice. Cancer Res. 13: 590–592, 1953.

(*29*) BERNFELD, P., JACOBSON, S., and BERNFELD, H. C.: "Activator-competitive" enzyme inhibition. Interrelation of dissociation, activation, inhibition and surface inactivation of β-glucuronidase. Arch. Biochem. 69: 198–209, 1957.

(30) DE DUVE, C.: Lysosomes, a new group of cytoplasmic particles. *In* Subcellular Particles (Hayashi, T., ed.). New York, The Ronald Press Company, 1959, pp. 128–159.

(31) THOMSON, J. F., and KLIPFEL, F. J.: Further studies on cytoplasmic particulates isolated by gradient centrifugation. Arch. Biochem. 70: 224–238, 1957.

(32) DE DUVE, C., BEAUFAY, H., JACQUES, P., RAHMAN-LI, Y., SELLINGER, O. Z., WATTIAUX, R., and DE CONINCK, S.: Intracellular localization of catalase and of some oxidases in rat liver. Biochim. et biophys. acta 40: 186–187, 1960.

(33) MILLS, G. T., and SMITH, E. E. B.: The β-glucuronidase activity of chemically induced rat hepatoma. Science 114: 690–692, 1951.

(34) MILLS, G. T., PAUL, J., and SMITH, E. E. B.: Studies on β-glucuronidase 3. The influence of age, partial hepatectomy and other factors on the β-glucuronidase activity of rat liver. Biochem. J. 53: 245–253, 1953.

(35) WESTFALL, B. B., EVANS, V. J., SHANNON, J. E., JR., and EARLE, W. R.: The glycogen content of cell suspensions prepared from massive tissue culture: Comparison of cells derived from mouse connective tissue and mouse liver. J. Nat. Cancer Inst. 14: 655–664, 1953.

(36) PEPPERS, E. V., WESTFALL, B. B., and EARLE, W. R.: Glycogen content of cell suspensions prepared from massive tissue culture. II. Comparison of fourteen cell strains after long cultivation *in vitro*. J. Nat. Cancer Inst. 23: 823–831, 1959.

(37) WESTFALL, B. B., PEPPERS, E. V., EVANS, V. J., SANFORD, K. K., HAWKINS, N. M., FIORAMONTI, M. C., KERR, H. A., HOBBS, G. L., and EARLE, W. R.: The arginase and rhodanese activities of certain cell strains after long cultivation *in vitro*. J. Biophys. & Biochem. Cytol. 4: 567–570, 1958.

(38) PEPPERS, E. V., WESTFALL, B. B., KERR, H. A., and EARLE, W. R.: Note on the catalase activity of several mammalian cell strains after long cultivation *in vitro*. J. Nat. Cancer Inst. 25: 1065–1068, 1960.

(39) SATO, G., ZAROFF, L., and MILLS, S. E.: Tissue culture populations and their relation to the tissue of origin. Proc. Nat. Acad. Sc. 46: 963–972, 1960.

(40) SELIGMAN, A. M., TSOU, K. C., RUTENBURG, S. H., and COHEN, R. B.: Histochemical demonstration of β-d-glucuronidase with a synthetic substrate. J. Histochem. & Cytochem. 2: 209–229, 1954.

(41) FISHMAN, W. H., and BAKER, J. R.: Cellular localization of β-glucuronidase in rat tissues. J. Histochem. & Cytochem. 4: 570–587, 1956.

(42) GOLDFARB, B., and BARKA, T.: Cytologic localization of β-glucuronidase. J. Histochem. & Cytochem. 8: 226–227, 1960.

(43) STRAUS, W.: Rapid cytochemical identification of phagosomes in various tissues of the rat and their differentiation from mitochondria by the peroxidase method. J. Biophys. & Biochem. Cytol. 5: 193–204, 1959.

(44) ———: Concentration of acid phosphatase, ribonuclease, deoxyribonuclease, β-glucuronidase, and cathepsin in "droplets" isolated from the kidney cells of normal rats. J. Biophys. & Biochem. Cytol. 2: 513–521, 1956.

Studies on the Exchange of Fluids Between Host and Tumor. I. A Method for Growing "Tissue-Isolated" Tumors in Laboratory Animals [1, 2]

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SUMMARY

A technique is described which provides the investigator with a growing tumor enveloped in paraffin and thus isolated from the surrounding tissues but connected with the host by a single artery and vein from which blood can be withdrawn. The procedure can be applied to all tumors normally grown in a solid form in rats, hamsters, and mice. Two transplants of the same tumor, one grown isolated in the paraffin envelope and the other grown subcutaneously, showed the same correlation between wet weight, dry weight, and total nitrogen, as well as the same level of anaerobic glycolysis. Animals bearing the "isolated" transplants behaved in the same manner as those bearing subcutaneous tumors in regard to body weight, food consumption, and water intake; liver catalase was also equally depressed.—J. Nat. Cancer Inst. 27: 679–693, 1961.

THE PROPERTIES of neoplasms have been studied by the analyses of the tumor-bearing host, fragments of the tumor, the tumor cells, or cell particles. To our knowledge a technique does not exist which allows the study of the tumor as a mass of growing tissue isolated from the surrounding organs and connected with the host by only a tiny peduncle bearing the vessels. A technique, which fulfills this purpose has been developed in this laboratory (*1*). This procedure has proved useful in studies on the blood supply of tumors and promises to have wide application to problems regarding the tumor-host relationship.

MATERIALS AND METHODS

Tumors of 12 different types were implanted in adult rats, hamsters, and mice (table 1). Ether or Nembutal (35 mg/kg) was used as an anesthetic, and surgical procedures were carried out with the usual precautions. One

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or 2 drops of crysticillin 600 AS [5] were placed in the surgical field after each treatment to prevent infection.

TABLE 1.—Tumors grown "tissue-isolated" in a paraffin bag

Host	Strain	Tumor	Carcinogen	Reference
Rat	Buffalo (inbred)	Hepatoma 5123	*N*-2-fluorenylphthalamic acid	(*2*)
Rat	Fischer 344 (inbred)	Hepatoma LC18	*N*-2-fluorenylacetamide	(*3*)
Rat	Sprague-Dawley	Novikoff hepatoma	4-Dimethylaminoazobenzene	(*4*)
Rat	A × C	Hepatoma 3683	*N*-2-fluorenyldiacetamide	(*5*)
Rat	Osborne-Mendel (inbred)	Ethionine hepatoma	Ethionine	(*6*)
Rat	Sprague-Dawley	Walker carcinosarcoma 256	"Spontaneous"	(*7*)
Rat	M520 × A × C	Lymphosarcoma R2788	"Spontaneous"	(*8*)
Rat	Buffalo (inbred)	Fibrosarcoma 4956	*N*-4-(4′-fluorobiphenyl)-acetamide	(*)
Hamster	Syrian-Golden	Angiosarcoma †	4-Dimethylaminoazobenzene	(*9*)
Hamster	Syrian-Golden	Cholangiocarcinoma†	*N*-2-fluorenylacetamide	(*10*)
Mouse	C3H/f	Osteogenic sarcoma	"Spontaneous"	(‡)
Mouse	C3H/HeN	Mast-cell neoplasm	Methylcholanthrene	(*11*)

*Obtained from Dr. H. P. Morris, National Cancer Institute.
†Obtained from Dr. P. Shubik, Chicago Medical School.
‡Obtained from Dr. K. C. Snell, National Cancer Institute

Isolation of the Host Organ

The vascular connections of the kidney and the ovary in a rat are represented in text-figure 1. The most common variation involves the left ovarian artery, which sometimes branches from the renal artery. A less frequent variation is the entry of the left ovarian vein into the inferior vena cava somewhere between the renal and the iliolumbar vein.

The peritoneal cavity was reached through an incision of about one centimeter in the left lumbar region. The kidney was carefully isolated from the surrounding adipose tissue and pulled out through the muscle layers into the subcutaneous space. The left kidney was always chosen because its vascular peduncle is longer. The muscular layers were not sutured too tightly to avoid immediate cyanosis and late hydronephrosis.

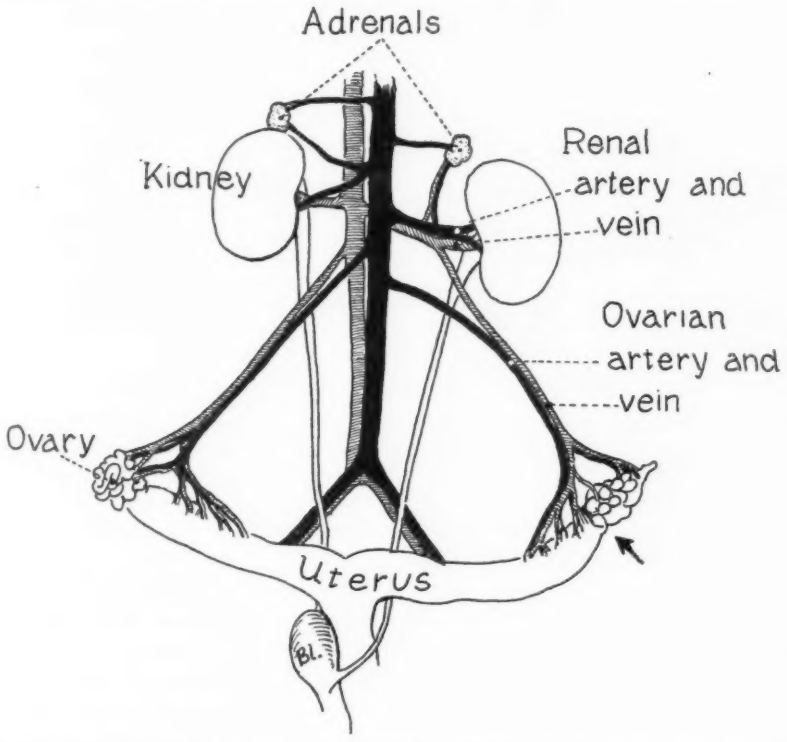
The same procedure can be used for the isolation and displacement of the ovary after the oviduct has been separated from the uterus and the mesovarium has been cauterized up to the origin of the ovarian vessels (text-fig. 2).

Transplantation of the Tumor

An hourglass-shaped paraffin sheet [6] was prepared with a slit in the lower half (text-fig. 3*A*). The vascular peduncle of the kidney or the

[5] E. R. Squibb and Sons, New York, N.Y.
[6] Parafilm "M"—Marathon Division, American Can Co., Menasha, Wis.

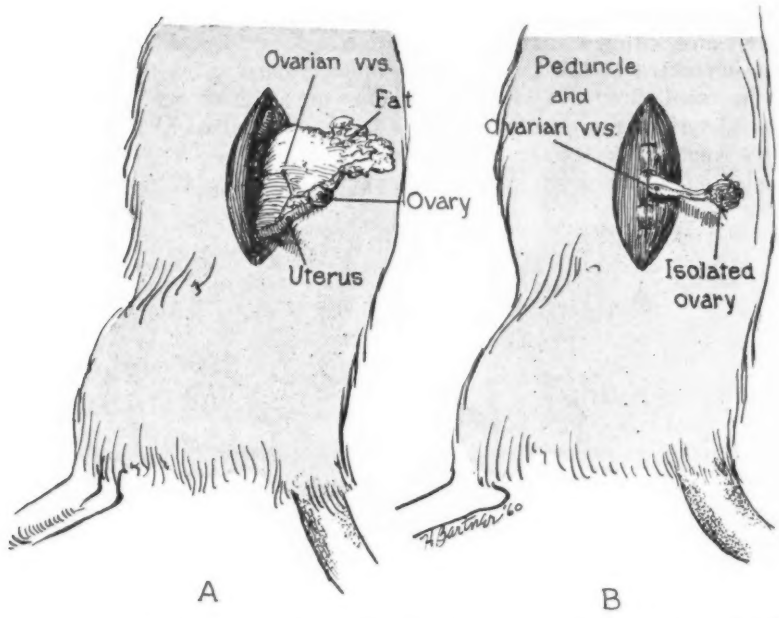
ovary was placed in the slit and the edges of the slit were overlapped and heat-sealed with a soldering gun to form a cup (text-fig. 3*B*). The tumor was implanted at this time. The neoplastic mass taken from the host was pressed through a mesh of 0.7 mm pore diameter and placed in a special syringe represented in figure 1. This syringe was developed to force fragments of tumors through a small needle required for accurate implants in small organs such as the ovary of the rat. The total amount



TEXT-FIGURE 1.—Vascular connections of kidneys and ovaries in a rat. *Arrow* points to area where the surgical separation between uterus and ovary was performed.

of tissue injected varied from about 60 mg in the kidneys to 10 mg or less in the ovary. Hemorrhage from the host organ could not always be avoided.

After the tumor was implanted, a drop of crysticillin was placed on the host organ, and the second part of the paraffin sheet was folded onto the cup and melted to form a sealed envelope (text-fig. 3*C*). The envelope was then placed in a pouch of the subcutaneous tissue and the skin was sutured with nickel-silver clips. After this stage the host organ, implanted with the tumor, was contained in a sealed bag of paraffin which allowed only the passage of the vascular peduncle.



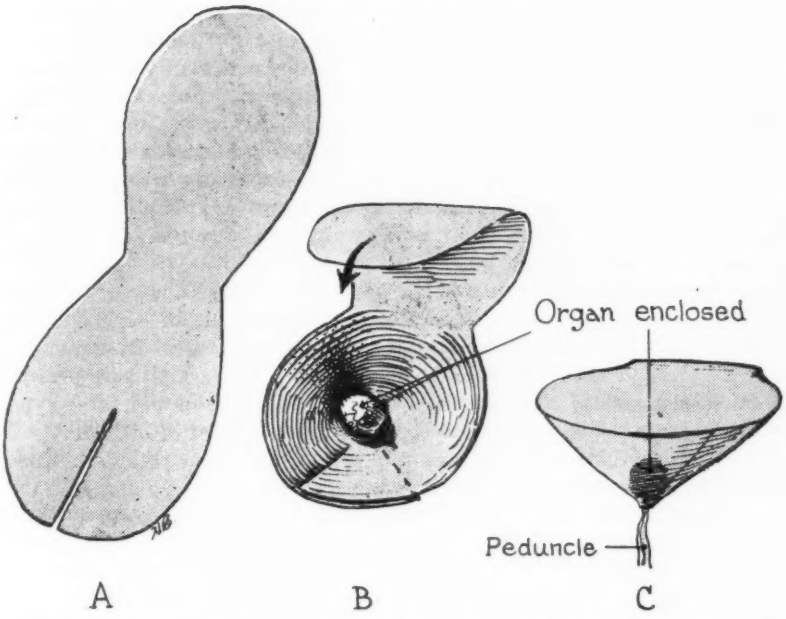
TEXT-FIGURE 2.—In *A*, the ovary, uterus, and mesovarium were pulled out of the abdominal cavity. In *B*, the uterus was separated from the oviduct, and the mesovarium cauterized to form a peduncle for the ovarian vessels.

Isolation and Care of the Tumor

Five or 6 days after implantation, the pouch of the skin was opened, the paraffin sack discarded, and the size of the tumor measured. The pouch usually contained clotted fibrin and a few milliliters of liquid resembling blood plasma, sometimes very rich in erythrocytes. The peduncle was from 1 to 3 cm long and the connective tissue surrounding the vessels was often markedly edematous. To avoid spread of the tumor it was very important at this stage to dissect the connective tissue of the peduncle and leave the blood vessels as "naked" as possible. The edema made the dissection easier. A new paraffin envelope was made and the skin resutured. In fast-growing tumors a single replacement was sufficient, but in slow-growing tumors it was necessary to repeat the replacement procedure every 10 days. The results obtained with the technique described are shown in figures 2 and 3.

SOME BIOLOGIC CHARACTERISTICS OF "TISSUE-ISOLATED" TUMORS IN RATS

(*a*) *Macroscopically* the tumors grown tissue-isolated resembled the usual subcutaneous transplants. The shape of the tumor was most



TEXT-FIGURE 3.—(*A*) An hourglass-shaped paraffin sheet with a slit in one half; (*B*), paraffin sheet placed around the renal or ovarian peduncle to form a cup; (*C*) upper flap folded and heat-sealed over cup to form a bag.

frequently round or ovoid. The consistency varied with the type of tumor, but it was always sufficiently firm to permit easy handling of the neoplastic mass. The color varied from white to the dark red of the hemorrhagic areas, often in the same tumor; yellowish sectors with the macroscopic and microscopic aspect of the anemic infarct were sometimes found in slow-growing tumors like Hepatoma 5123. The surface of both the renal and ovarian implants was always smooth; sometimes it was even, and sometimes it was covered with small buds. On cross section, the necrotic areas appeared scattered all over the surface; however, none of the tumors implanted showed the central necrotic portion frequently seen in subcutaneous transplants. The over-all impression was that tumors grown in paraffin bags had less extensive necrotic areas than the subcutaneous transplants of equal size.

Measurement of the diameters of tumors without interference of any other tissue became possible when the tumors were removed from the subcutaneous pouches and the paraffin envelopes were discarded.

(*b*) *The destruction of the host organ* was followed by histologic sectioning. In kidney implants, even when the tumor was 15-fold larger than the opposite kidney, it was possible to find some intact glomeruli or tubuli dispersed in the neoplastic tissue and surprisingly well-preserved. In some of these tumors a ligature of the ureter still permitted the col-

lection of small amounts of urine. On histologic sectioning of ovarian implants, ovarian follicles have been found in 4 g tumors, which corresponds to about a 100-fold increase in weight over that of the host ovary. They were not found in larger tumor implants. Sometimes ovarian implants contained cysts full of a clear liquid under tension. The wall of the cyst was formed by connective tissue, ovarian tissue, and tumor cells. If a cyst was opened, in a few days the cavity was invaded by neoplastic cells and the ovarian tissue was destroyed. If a cyst was not opened it became smaller as the tumor grew and usually disappeared when the tumor was very large.

It seems valid to say that tumor implants of the kidney, even when they grow to 15 g, probably contain varying amounts of normal tissue, while tumors grown in the ovary and exceeding 4 g are free from morphologically recognizable tissue of the host organ when no cysts are present.

(*c*) *The growth of each neoplasm* can be compared in the three types of transplants by implanting the same amount of tumor into three groups of animals and weighing the tumor masses after equal periods of time. The conditions of growth, however, cannot be the same for all three transplants because the animal with a tissue-isolated tumor must undergo a surgical treatment when the paraffin envelope of the neoplasm is changed. Despite this important limitation, the tumors studied can be divided into two groups and classified as "fast-" or "slow-" growing, both in tissue-isolated and subcutaneous transplants. The first group includes Novikoff hepatoma, Hepatoma 3683, Hepatoma LC18, Walker carcinosarcoma 256, and Lymphosarcoma 2788. The second group includes Hepatoma 5123 and Fibrosarcoma 4956.

Novikoff hepatoma proved the fastest growing tumor of the group. At the 6th day after transplantation, about 3 g of tumor were produced in the ovary and 2 g in the kidney, while the subcutaneous transplants reached this size only at the 8th day. During the 2d week the growth became explosive. At the 8th day the average weight of ovarian implants was 6.4 $\pm$ 0.5 g and at the 12th day 12.8 $\pm$ 1.7. The renal transplants at the 9th day weighed 6.0 $\pm$ 0.5 g and at the 12th day 9.2 $\pm$ 1.2 g. The subcutaneous transplants grew slower: 2.2 $\pm$ 0.5 at the 8th day, 3.6 $\pm$ 0.7 at the 11th day, and 6.1 $\pm$ 0.8 at the 12th day.

Among the other fast-growing tumors, Hepatoma 3683, Hepatoma LC18, and Walker carcinosarcoma 256 followed this pattern: At the 12th day the ovarian transplants weighed, respectively, 6.5 $\pm$ 0.5 g, 6.1 $\pm$ 0.8 g, and 8.6 $\pm$ 0.8 g; the renal transplants 6.0 $\pm$ 0.5 g, 5.7 $\pm$ 0.9 g, and 7.3 $\pm$ 0.9; the subcutaneous transplants 5.6 $\pm$ 0.6 g, 5.8 $\pm$ 0.9, and 6.2 $\pm$ 0.6. Lymphosarcoma R2788 at the 11th day weighed 5.6 $\pm$ 0.6 g in the ovary, 8.0 $\pm$ 0.5 g in the kidney, and 4.5 $\pm$ 0.8 in the subcutaneous transplants.

The average weight of renal implants of Hepatoma 5123 at the 40th day of transplantation was 6.4 $\pm$ 0.4, while at this time the ovarian and subcutaneous transplants weighed roughly one half as much. An average weight of 7.1 $\pm$ 1.0 g was obtained in renal implants of Fibrosarcoma

4956 at the 21st day after implantation; the ovarian implants weighed about one half as much at this time, and the subcutaneous transplants two thirds as much.

The source of the material used for transplanting influenced the growth of the tissue-isolated tumors. It has been observed that Novikoff hepatoma, Hepatoma 3683, Walker carcinosarcoma 256, and Lymphosarcoma R2788 grew faster if the donor tumor had been grown intraperitoneally rather than subcutaneously.

Although the experimental conditions differed in the three types of transplants, the results reported in this section give the over-all impression that "tissue-isolated" tumors grow as well as, or better than, subcutaneous transplants. The evaluation of growth was limited to relatively small tumors because the larger masses of neoplastic tissue could not be kept "tissue-isolated."

(*d*) *The relationship between wet weight, dry weight, and total nitrogen* was studied in tissue-isolated and subcutaneous implants of Novikoff hepatoma, Hepatoma 3683, Hepatoma LC18/81A, and Walker carcinosarcoma 256. The tumors were implanted subcutaneously in one group of rats, in the kidney in a second group, and in the ovary in a third group; males and females were used in the first two groups. The animals were killed when the tumors weighed 1.0 to 12.0 g. No significant difference was found among tumors grown with the three different techniques.

(*e*) *The glycolytic activity* and the effect thereon of testosterone were examined by the procedure of Woods *et al.* (*12*) in ovarian and subcutaneous implants of Novikoff hepatoma and Walker carcinosarcoma 256. Each animal received both implants, the subcutaneous first and, after a few days, the ovarian. The glycolytic activity was of the same order of magnitude for the ovarian and the subcutaneous implants of both tumors. Glycolysis was not inhibited by testosterone in both transplants of Walker carcinosarcoma 256 and was slightly reduced in both transplants of Novikoff hepatoma.

(*f*) *The effect on the host of tissue-isolated transplants* was studied by measurement of food intake, water consumption, and body-weight change in three groups of rats each bearing, respectively, the subcutaneous, renal, or ovarian implant.

Novikoff hepatoma, Hepatoma 3683, Hepatoma LC18/81A, and Lymphosarcoma R2783 were grown for a suitable period in rats of the same age and initial body weight that were kept on a normal Purina chow diet. Table 2 shows the differences between body weight, food intake, and water consumption of rats with subcutaneous and those with tissue-isolated transplants.

The increased water consumption of rats bearing tissue-isolated tumors as compared to the intake of rats with subcutaneous tumors was the most marked difference observed. This was especially evident in the last days of the experiment. The large amount of fluid found in the subcutaneous pouches of rats bearing isolated tumors may be a partial

TABLE 2.—Body weight, food intake, and water consumption of untreated rats bearing subcutaneous and "tissue-isolated" transplants*

Tumors:		Novikoff hepatoma	Hepatoma 3683	Hepatoma LC18	Lymphosarcoma R2788
Days of experiment:		14	15	16	16
Total gain in body weight (g)	Untreated rats	+22	+20	+8.	+55
	Implants: subcutaneous	+25	+14	+2. 8	+34
	Implants: ovarian	+22	+20	+7. 8	+65
	Implants: renal	+19	+ 6	+2. 5	+55
Food consumption (g/day)	Untreated rats	13. 0 ± 0. 4	9. 4 ± 0. 2	8. 6 ± 0. 2	13. 1 ± 0. 3
	Implants: subcutaneous	14. 0 ± 0. 3	9. 0 ± 0. 3	8. 0 ± 0. 2	8. 7 ± 0. 3
	Implants: ovarian	11. 6 ± 0. 7	8. 9 ± 0. 4	9. 0 ± 0. 4	10. 8 ± 0. 4
	Implants: renal	10. 2 ± 0. 8	7. 5 ± 0. 5	6. 9 ± 0. 4	9. 9 ± 0. 5
Water consumption (ml/day)	Untreated rats	22. 3 ± 0. 8	10. 7 ± 0. 3	12. 8 ± 0. 3	14. 7 ± 0. 2
	Implants: subcutaneous	21. 3 ± 0. 5	11. 2 ± 0. 5	13. 1 ± 0. 7	14. 8 ± 0. 4
	Implants: ovarian	23. 9 ± 1. 6	13. 8 ± 0. 3	15. 5 ± 0. 4	20. 6 ± 0. 5
	Implants: renal	26. 9 ± 1. 4	14. 8 ± 0. 6	14. 9 ± 0. 3	26. 2 ± 0. 7

*The ± means ± standard errors on groups of 5 animals, 150 to 200 g body weight at time of tumor implantation.

explanation of this increased water intake. About 7 days after the tumors were transplanted an increasingly large amount of hemorrhagic fluid appeared between the skin and the paraffin bags. The fact that the amount of fluid in the pouch was negligible when the kidney or the ovary was enveloped alone would indicate that the fluid was related to the presence of the tumor. The presence of clotted blood in this fluid suggests hemorrhage as an etiologic factor. The hemorrhage occurred more frequently in the renal than in the ovarian transplants, and it was more often associated with Hepatoma 3683 than with other tumors. The increased frequency of hemorrhage in rats bearing renal transplants may also partially account for the decreased food consumption of these animals.

(*g*) *Liver catalase activity* was measured in rats bearing implants of various types and sizes. Catalase was assayed spectrophotometrically by the method of Beers and Sizer (*13*), as modified by Price and Greenfield (*14*, *15*). The tissue-isolated transplants caused a marked reduction in liver catalase and the highest reduction corresponded to the largest tumors (table 3).

The findings are in accordance with the data reported in the literature for subcutaneous transplants of various types of tumors (*16*).

TABLE 3.—Liver catalase activity of rats bearing host-isolated tumors

	Tumor-bearing rats			Controls*
Rat No.	Tumor	Weight of tumor	Liver catalase†	Liver catalase†
1	Novikoff hepatoma ‡	5. 4	957	1178
2	"	5. 8	383	
3	"	10. 2	480	
4	"	14. 9	238	
5	Hepatoma LC18§	5. 0	196	817
6	"	6. 8	60	
7	"	9. 1	46	
8	"	9. 3	55	

*Livers of 5 animals of the same weight range were pooled.

†Total amount of catalase activity in the whole liver of animals of comparable size. [Numbers represent units according to (*14*, *15*).]

‡Sprague-Dawley rats weighing 180 to 200 g. Tumors implanted in the kidney.

§Fischer 344 rats, weighing 125 to 140 g. Tumors implanted in the ovary.

DISCUSSION

The technique described offers two major advantages: (*a*) The tumor grows isolated from the surrounding tissues, and (*b*) it is connected to the host with only one artery and one vein. Thus, studies can be carried out on the composition of the tumor with minimum contamination by extraneous tissues; in fact, the paraffin envelope avoids any invasion of the surrounding tissues. Also, the amount of tumor grown may be very large when compared with the host organ, as much as 400-fold greater with

ovarian implants. The connection through the tiny peduncle bearing one artery and one vein offers an ideal condition for the study of the exchange of fluids between host and tumor. Samples of blood going in and out of the tumor can be taken at different intervals and, when the artery and the vein are cannulated, the tumor can be perfused without leakage.

The "host-isolated" and subcutaneous transplants did not differ appreciably when the water and nitrogen content or the glycolytic activity was tested. The relationship between host and tumor, evaluated by comparing body-weight change, food intake, water consumption, and depression of liver catalase, was unaffected by the technique. It seems safe to state that the experimental conditions described do not change the biological characteristics of the tumors as they are known in subcutaneous transplants or the response of the host.

REFERENCES

(*1*) Gullino, P. M.: Technique for growing in vivo "host-isolated" tumors. Fed. Proc. 20: 153, 1961.

(*2*) Morris, H. P., Sidransky, H., Wagner, B. P., and Dyer, H. M.: Some characteristics of transplantable rat hepatoma No. 5123 induced by ingestion of *N*-(2-fluorenyl)phthalamic acid. Cancer Res. 20: 1252–1254, 1960.

(*3*) Dunning, W. F., Curtis, M. R., and Maun, M. E.: The effect of added dietary tryptophane on the occurrence of 2-actylaminofluorine-induced liver and bladder cancer in rats. Cancer Res. 10: 454–459, 1950.

(*4*) Novikoff, A. B.: A transplantable rat liver tumor induced by 4-dimethylaminoazobenzene. Cancer Res. 17: 1010–1027, 1927.

(*5*) Morris, H. P.: Hepatocarcinogenesis by 2-acetylaminofluorene and related compounds including comments on dietary and other influences. J. Nat. Cancer Inst. 15: 1535–1545, 1955.

(*6*) Farber, E.: Carcinoma of the liver in rats fed ethionine. A.M.A. Arch. Path. 62: 445–453, 1956.

(*7*) Stewart, H. L., Snell, K. C., Dunham, L. J., and Schlyen, S.M.: Transplantable and Transmissible Tumors of Animals. *In* Atlas of Tumor Pathology, Section XII, Fascicle 40. Washington, D.C., Armed Forces Institute of Pathology, 1959, p. 261.

(*8*) Curtis, M. R., and Dunning, W. F.: Transplantable lymphosarcomata of the mesenteric lymph nodes of rats. Am. J. Cancer 40: 299–309, 1940.

(*9*) Terracini, B., Della Porta, G., and Maxwell, S.: A transplantable hemoangiosarcoma of the Syrian golden hamster. Transpl. Bull. 6: 432–435, 1959.

(*10*) Della Porta, G., Shubik, P., and Scortecci, V.: The action of *N*-2-fluorenylacetamide in the Syrian golden hamster. J. Nat. Cancer Inst. 22: 463–488, 1959.

(*11*) Dunn, T. B., and Potter, M.: A transplantable mast cell neoplasm in the mouse. J. Nat. Cancer Inst. 18: 587–601, 1957.

(*12*) Woods, M., Hunter, J., and Burk, D.: Regulation of glucose utilization in tumors by a stress-modified insulin : anti-insulin system. J. Nat. Cancer Inst. 16: 351–404, 1955.

(*13*) Beers, R. F., Jr., and Sizer, I. W.: A spectrophotometric method measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. 195: 133–140, 1952.

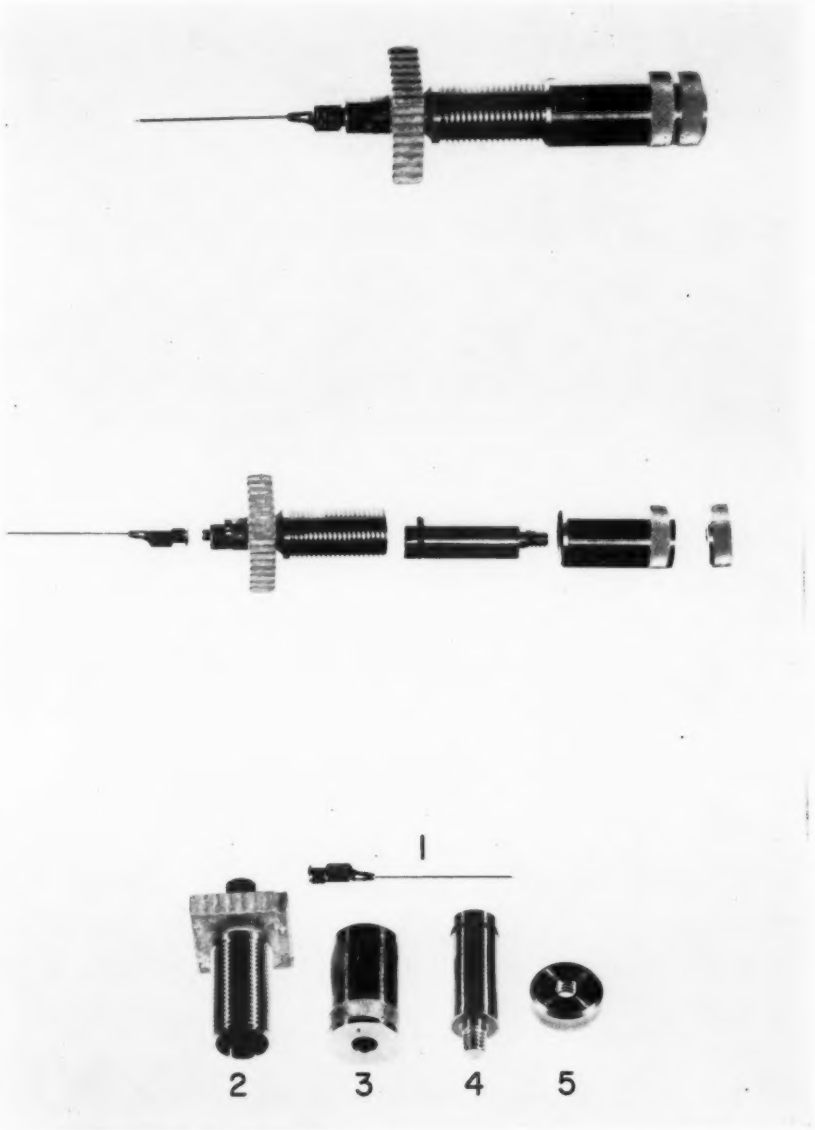
(*14*) Price, V. E., and Greenfield, R. E.: Anemia in cancer. Advances Cancer Res. 5: 199–284, 1958.

(*15*) GREENFIELD, R. E., and PRICE, V. E.: Liver catalase. III. Isolation of catalase from mitochondrial fractions of polyvinylpyrrolidone-sucrose homogenate. J. Biol. Chem. 220: 607–618, 1956.

(*16*) GREENSTEIN, J. P.: Biochemistry of Cancer, 2d. ed. New York, Academic Press, 1954, 519 pp.

PLATE 83

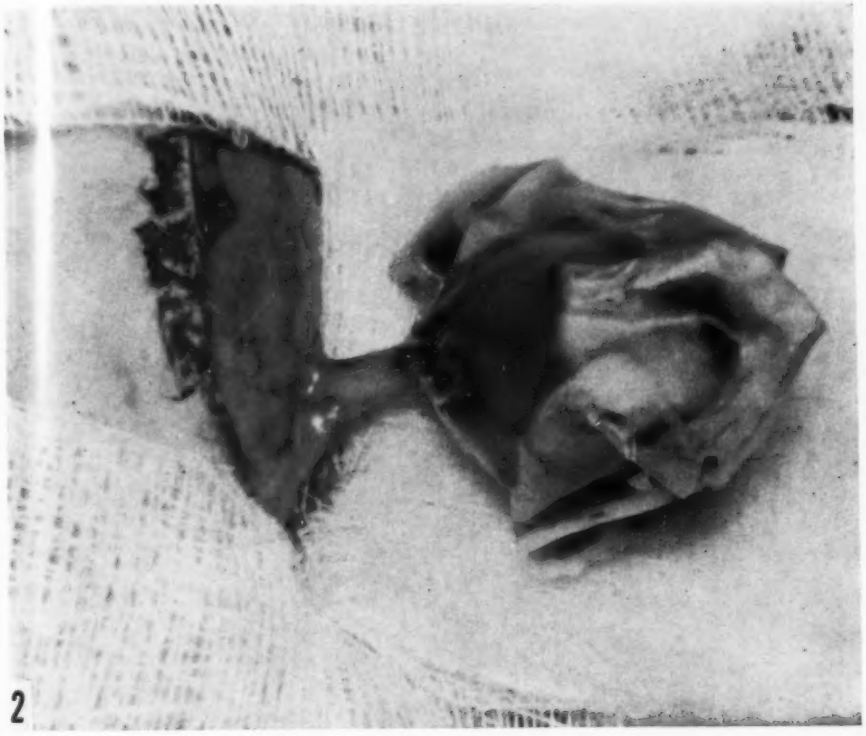
FIGURE 1.—Disarticulated stainless-steel syringe used for implanting tumor, in the process of being assembled. (1)—Needle #22. (2)—Cylinder containing fragments of tumor. (3)—Cylinder containing plunger (4) which is fixed by a screw (5). When cylinder #3 is turned, tumor fragments in cylinder #2 are pushed through needle. Plunger (4) has a rubberband gasket which prevents backflow of tumor slurry.



1

PLATE 84

FIGURES 2 and 3.—Novikoff hepatoma weighing 4.4 g implanted in ovary 6 days previously. Tumor has been removed from subcutaneous pouch and is shown with and without paraffin sack. *Note* edema of peduncle and the complete isolation afforded by bag.



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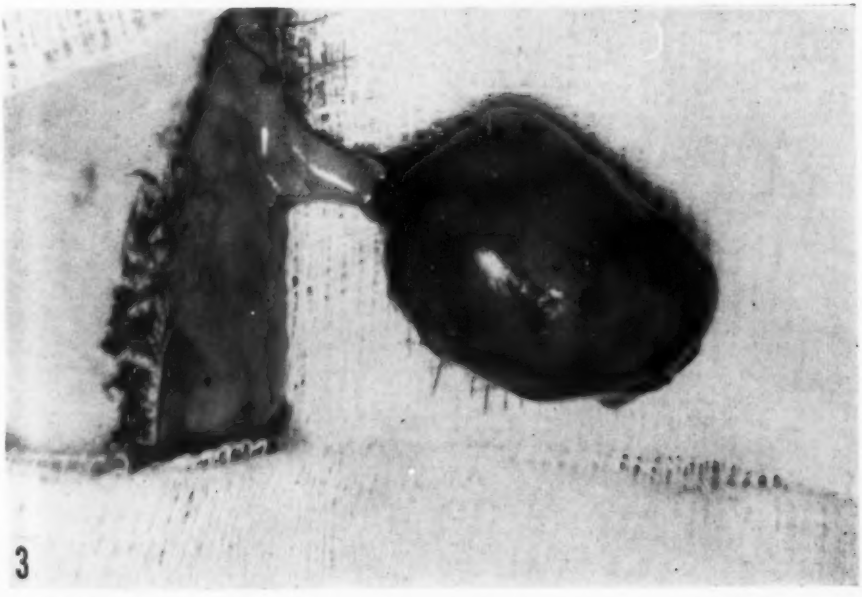

3

Comparative Study of the Carcinogenic Activity of Nitrosamines [1, 2]

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SUMMARY

The carcinogenic activity of dimethyl-, diethyl-, and diphenylnitrosamine and dimethylhydrazine was studied in the rat. The high carcinogenic activity of dimethylnitrosamine and diethylnitrosamine was confirmed; the localization and morphology of the tumors differed from those previously described. Diphenylnitrosamine and dimethylhydrazine had no carcinogenic activity. Dimethylnitrosamine produced tumors with a variety of histological characteristics in the liver, lung, and kidney. Primary malignant tumors arose in 1, 2, or all 3 of these organs of the same rat. In the lung, squamous-cell carcinomas were found either as the only pulmonary tumor or in association with bronchiolar or alveolar adenocarcinoma. Diethylnitrosamine induced hepatocellular carcinomas in all rats, with metastases into the lung in many instances. One primary kidney tumor was found.—J. Nat. Cancer Inst. 27: 695–709, 1961.

IN THE course of investigating the causes of liver damage in two industrial workers, Barnes and Magee in 1954 demonstrated that dimethylnitrosamine (DMN) provokes liver injury in rats (*1*). When this substance was fed for many months, tumors were produced in the liver (*2*); later, tumors in the kidney and in the lung were also observed (*3*). It was reported that primary liver and kidney tumors did not occur together in the same rat. A similar impression could be gained from the work of Zak *et al.* (*4*) in which the production of kidney and lung tumors in rats by DMN was reported, but in the liver only "hyperplastic and cystic nodules could be observed in a relatively high incidence."

While experiments on the effect of structural changes in the nitrosamine molecule on the carcinogenic and metabolic activities of these substances were in progress, a note was published (*5*) on the production of cirrhosis

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[4] The authors wish to thank Mrs. Judith F. Kane for her expert assistance, and Dr. Joseph C. Arcos for helpful discussion.

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and cancer of the liver in rats by diethylnitrosamine (DEN) fed in drinking water. Hemorrhages in the lungs, thymus, para-aortic lymph nodes, and the gastric mucosa were also observed.

In the present paper the comparative carcinogenicity and tissue specificity of DMN, DEN, diphenylnitrosamine (DPhN), and dimethylhydrazine (DMH) are reported.

MATERIALS AND METHODS

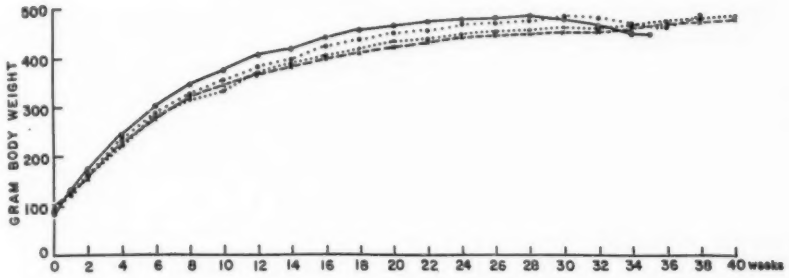
The nitrosamines and dimethylhydrazine were purchased from the Eastman-Kodak Company. Equimolar amounts of DMN (400 μg), DEN (550 μg), DPhN (1070 μg), and DMH (325 μg) were given daily 5 times a week in 1 ml water by stomach tube, except DPhN which was suspended in 1 ml of 1 percent aqueous methylcellulose. It was shown in previous experiments that methylcellulose is not carcinogenic and does not interfere with the carcinogenic activity of another compound (*6*). Solutions or suspensions were prepared fresh daily just before administration.

Male Wistar rats supplied by Carworth Farms were used, 25 per experimental group. They weighed on the average 92 g at the beginning of the experiment and were maintained on Purina laboratory chow and water *ad libitum*. The experiment with DMN was terminated between 35 and 48 weeks, that with DEN between 22 and 35 weeks, and that with DPhN and DMH in the 53d week; the feeding of the two latter substances was discontinued after 45 weeks. Animals that were killed or died on or after the 22d week and all those killed at the end of the experiments were autopsied, and portions of liver, spleen, kidneys, lung, and any other organs with macroscopic changes were fixed in Mossman's fixative. Hematoxylin-eosin stain was used routinely; mucicarmine, periodic acid-Schiff, or aldehyde fuchsin stains were employed when indicated.

RESULTS

Text-figure 1 shows that the average weight of the rats receiving DMN or DEN was slightly above that of the rats on DPhN or DMH until 30 weeks. After that time the average weights in the groups receiving DMN or DEN declined slowly. Some individual animals lost considerable weight. The slight increase in the average weight of rats on DMN after the 36th week may have been caused by ascites or an increase in the size of the tumor.

The duration of the experiment, the amount of nitrosamine derivatives and DMH given, and the tumor incidence are shown in table 1. Twenty-two of 25 rats, receiving DMN lived longer than 31 weeks. In 1 animal, found dead in the 34th week, a tumor was seen in the liver on gross examination, but no microscopic investigation was performed. The histopathological findings in 21 rats are summarized in table 2. All rats



TEXT-FIGURE 1.—Growth curves of rats fed nitrosamine and hydrazine derivatives.

Rats fed dimethylnitrosamine ●.....●
Rats fed diethylnitrosamine ○———○
Rats fed diphenylnitrosamine X-------X
Rats fed dimethylhydrazine ▲.....▲
Each point represents the average weight of 22 to 25 rats.

TABLE 1.—Carcinogenic activity of nitrosamine derivatives and dimethylhydrazine to rats

	Duration of experiment (weeks)	Total amount per rat (mg)	Rats with tumor/ rats in experiment
$(CH_3)_2N{\cdot}NO$, DMN	31–48	62–96	22/22*
$(C_2H_5)_2N{\cdot}NO$, DEN	22–35	61–96	25/25
$(C_6H_5)_2N{\cdot}NO$, DPhN	53†	244	0/25
$(CH_3)_2N{\cdot}NH_2$, DMH	53†	74	0/25

*In 1 rat a tumor was seen in the liver but not examined microscopically.
†Compound given for 45 weeks.

TABLE 2.—Location and histological type of tumors produced by DMN

		Predominant type of tumor							
		Liver			Kidney			Lung	
	Number of rats	Liver cell	Bile duct	Sarcoma	Adenocarcinoma	Solid carcinoma	Sarcoma	Squamous cell	Adenocarcinoma
Single primary	8								
Liver	3	2		1					
Kidney	1						1		
Lung	4							2	2
Two primaries	7								
Liver and kidney	2	1	1		1	1			
Liver and lung	2		2					1	1
Lung and kidney	3				2	1		1	2
Three primaries	6								
Liver, kidney, lung	6	4	2		4	2			6

developed tumors, which were localized in the liver, kidney, or lung. Besides the primary tumors, metastatic tumors from the liver were found in the lungs. The diagnosis of multiple primary tumors was based on the usually accepted criteria: marked differences in the morphology of the tumors and demonstrations of specific metabolic products in the cells or the transformation of normal to neoplastic epithelium. No organs apart from the liver, lung, or kidney were involved in the neoplastic process. In 6 rats primary tumors were in all 3 locations, in 7 rats 2 primary sites were observed, and in 8 rats tumors in a single location were found.

The organs involved were grossly much enlarged and had multiple, pale, elevated nodules. In the liver the nodules reached several cm in diameter; in the lungs and kidneys they rarely exceeded 1 cm. Occasionally the tumors in all three locations appeared cystic with a dark-yellow or slightly hemorrhagic content. On histological investigation the tumors of the liver consisted either of long, 3 to 5 cells thick, finger-like strands of large, cytoplasm-rich cells, which produced the pattern of hepatocellular carcinomas (fig. 1) or of glandular arrangements of cuboidal or columnar cells showing secretion and often containing bile pigment (fig. 2). Both types of tumor were occasionally in the liver of the same animal. Invasion of the vessels by the tumor could be observed in some instances. In 1 rat the tumor consisted of long, spindle-shaped cells with abundant intercellular substance and was diagnosed as a fibrosarcoma (figs. 3 and 4). Sarcomatous elements were found in the stroma of another tumor with predominantly cholangiocarcinomatous features. No cirrhosis was found, but multiple foci of necrosis were present. Cells with hyperchromatic or bizarre nuclei and many mitoses were present in the non-neoplastic areas.

The tumors in the kidneys were mostly papillary adenocarcinomas, single or, more often, multiple, and measured grossly from 0.5 to 1.0 cm in diameter. The tumors seemingly originated from tubules and, in some instances, appeared to lie in cysts or dilated tubules (fig. 5). Solid tumors, formed by large, cytoplasm-rich cells, resembling clear cell carcinomas, were also observed (fig. 7). In one kidney, spindle-shaped cells with elongated nuclei and small round cells with sparse cytoplasm formed the bulk of the tumor. Interspersed epithelial structures resembling newly formed glomeruli and small tubules were found. From the histological similarity the lesion was designated as Wilms' tumor (fig. 9).

Of particular interest are the tumors that developed in the lung. Four squamous-cell carcinomas were found: in 2 animals as the only tumor in the body, and in the 2 others, simultaneously with primary liver or kidney tumors (figs. 10 and 11). The squamous-cell carcinomas were about 1.5 to 2 cm in diameter. Squamous metaplasia of the bronchial epithelium was demonstrated, and in 2 animals there was evidence of direct connection between the metaplastic epithelium and the carcinoma. In 1 animal both an adenocarcinoma and a squamous-cell carcinoma were present (fig. 8). Also, in 1 rat areas of squamous-cell carcinoma

were found in a predominantly adenomatous tumor. Adenocarcinoma occurred in 11 animals. This tumor was nearly always of multicentric origin. Most of the tumors were only a few millimeters large and were located in the periphery of the lung. They consisted of papillary or tubular arrangements of large, mostly columnar or cuboidal neoplastic cells (fig. 12). The tumors occurred in or were accompanied by areas of severe inflammation. Lesions—considered metastatic—were in 4 animals (figs. 4 and 6).

The tumors produced when DEN was fed were much more uniform than those produced by DMN. Five rats killed between the 22d and 35th weeks and 20 rats killed the 35th week all had liver tumors. On gross examination the left underlobe seemed most extensively involved. Cirrhosis was not found. Each tumor had the histological character of a hepatocellular carcinoma. Nine showed invasion of the small vessels of the lung and had multiple metastases in the parenchyma (figs. 13, 14, 15, and 16). In 1 animal a primary tubular carcinoma was in one kidney. Primary lung tumors were not seen. The hemorrhagic changes described by Schmähl *et al.* (*5*) were not observed.

DPhN and DMH were fed for 45 weeks and the rats killed in the 53d week. There were no tumors in any of these rats. The livers were normal grossly and microscopically; in many kidneys the tubules contained albuminous precipitation. Most lungs had peribronchial lymphocytic infiltration, as is very common in older rats; in some, squamous metaplasia of the bronchial epithelium was present, particularly in areas of bronchiectasis.

DISCUSSION

Although in the present experiment a striking difference was found in the location and morphology of the tumors produced by either DMN or DEN, the difference might have been caused in part by experimental conditions. The rats on DMN were kept alive longer than those on DEN. One kidney tumor was present in a rat fed DEN; therefore, in all likelihood more such tumors could be expected in a more prolonged experiment. No primary lung tumor was produced with DEN in this experiment, but it is possible that such tumors might have occurred under different conditions. The observation of Magee and Barnes (*3*) that liver and kidney tumors do not occur together in the same animal has not been confirmed in the present experiment.

The localization of the tumors produced by ingestion of DMN points to organs in which the chemical is either metabolized or excreted. Using C^{14}-labeled DMN, Dutton and Heath (*7*) showed in mice and rats that the main radioactive product is expired CO_2. In rats about 40 percent of the injected C^{14} is recovered by that route in 8 hours, 7 percent appears in the urine, and the remainder is fairly evenly distributed in the tissues, at the end of the experiment. The authors conclude that, because of the rapidity with which DMN is metabolized, the biologically active sub-

stance is a metabolite and not the DMN itself. Studies *in vitro* showed that DMN is rapidly metabolized by liver slices or homogenates, but little or not at all by other organs (*8*). Comparable studies on the metabolism of DEN are not known.

The production of squamous-cell carcinoma in the lung of rats by an *ingested* carcinogen is observed here for the first time. Lung tumors similar in morphological character and variety to those in the present experiment were produced by intratracheal inoculation of the polyoma virus to the Syrian golden hamster (*9*).

The type of relationship that has been formulated between electron density and the carcinogenic activity of the polycyclic hydrocarbons [reviewed *e.g.*, in (*10*)] may also hold for the nitrosamines, where carcinogenic activity is lost when the *N*-methyl or *N*-ethyl substituents are replaced by *N*-phenyl groups. Although the influence of steric hindrance, because of the relatively greater size of the phenyl groups, may not be excluded, circumstantial evidence suggests that these groups may actually function as electron traps redistributing the high electron density of the nitroso group. In fact, in both active compounds, DMN and DEN, the aminonitrogen has no basic properties in aqueous solutions, which indicates that there is a displacement of the free electron doublet of the nitrogen toward the nitroso group. The reactivity of the nitroso group, because of its high electron density, might then be instrumental in establishing the carcinogenic activity of the molecule following two possible mechanisms: (*a*) by altering vital metabolic control sites (*11*) or endoplasmic morphology (*12*), such as the nonmetabolized DMN or DEN, by virtue of their potent protein-denaturing ability (*13*); this denaturing ability has been shown to be in relation with the hydrogen-bonding ability of the nitroso group, which in turn is related to electron density. (*b*) by facilitating intramolecular cyclization of the *N*-monomethyl compound, an intermediate of metabolic breakdown (*14*), to diazomethane, which was shown to have carcinogenic activity (*15*); a similar mechanism may exist by analogy with DEN. It is also possible that situations (*a*) and (*b*) are not mutually exclusive, but are concurrently operative in living tissues.

The observations that DMN is toxic to the human liver and that nitrosamine derivatives are carcinogenic in experimental animals emphasize the danger of occupational and possibly nutritional exposure to this class of substances. Nitrosamine derivatives are still used industrially, particularly in Europe, in the vulcanization of rubber (*16*) and in the preparation of textile fibers (*17*).

REFERENCES

(*1*) Barnes, J. M., and Magee, P. N.: Some toxic properties of dimethylnitrosamine. Brit. J. Indust. Med. 11: 167–174, 1954.

(*2*) Magee, P. N., and Barnes, J. M.: The production of malignant primary hepatic tumours in the rat by feeding dimethylnitrosamine. Brit. J. Cancer 10: 114–122, 1956.

(3) ———: The experimental production of tumors in the rat. Acta Unio internat. contra cancrum 15: 187–190, 1959.

(4) Zak, F. G., Holzner, J. H., Singer, E. J., and Popper, H.: Renal and pulmonary tumors in rats fed dimethylnitrosamine. Cancer Res. 20: 96–100, 1960.

(5) Schmähl, D., Preussman, R., and Hamperl, H.: Leberkrebs erzeugende Wirkung von Diäthylnitrosamin nach oraler Gabe bei Ratten. Naturwissenschaften 47: 1–2, 1960.

(6) Argus, M. F., and Ray, F. E.: Possible relation between carcinogenicity and ease of hydrolysis *in vitro* of derivatives of 2-aminofluorene. Nature, London 184: 2018–2019, 1959.

(7) Dutton, A. H., and Heath, D. F.: Dimethylation of dimethylnitrosamine in rats and mice. Nature, London 178: 644, 1956.

(8) Magee, P. N., and Vandekar, M.: Toxic liver injury. The metabolism of dimethylnitrosamine *in vitro*. Biochem. J. 70: 600–605, 1958.

(9) Rabson, A. S., Branigan, W. J., and Legallais, F. Y.: Lung tumors produced by intratracheal inoculation of polyoma virus in Syrian hamsters. J. Nat. Cancer Inst. 25: 937–965, 1960.

(10) Pullman, A., and Pullman, B.: Electronic structure and carcinogenic activity of aromatic molecules. New developments. Advances Cancer Res. 3: 117–169, 1955.

(11) Arcos, J. C., Gosch, H. H., and Zickafoose, D.: Structural alterations in cell particles during chemical carcinogenesis. III. Selective action of hepatic carcinogens other than 3′-methyl-4-dimethylaminoazobenzene on different types of mitochondrial swelling. Effect of stimulated liver growth. J. Biophys. & Biochem. Cytol. In press.

(12) Emmelot, P., and Benedetti, E. L.: Changes in the fine structure of rat liver cells brought about by dimethylnitrosamine. J. Biophys. & Biochem. Cytol. 7: 393–396, 1960.

(13) Argus, M. F., Leutze, C. J., and Kane, J. F.: Nitrosamines and other carcinogens as agents of protein denaturation. Experientia. In press.

(14) Heath, D. F., and Dutton, A. H.: The detection of metabolic products from dimethylnitrosamine in rats and mice. Biochem. J. 70: 619–626, 1958.

(15) Schoental, R.: Carcinogenic action of diazomethoxane and of nitroso-*N*-methyl urethane. Nature, London 188: 420–421, 1960.

(16) Schultheiss, E.: Berufsdermatosen, Gummi und Ekzem, Editio Cantor, Aulendorf i. Wurtt., 1959, vol. 3, p. 213.

(17) Hueper, W. C.: Personal communication.

PLATE 85

FIGURE 1.—Hepatocellular carcinoma, rat fed DMN for 48 weeks. *Note* long strands of large cytoplasm-rich cells. Hematoxylin and eosin. × 150

FIGURE 2.—Cholangiocarcinoma, rat fed DMN for 37 weeks. Neoplastic cells are arranged in glandular pattern. Hematoxylin and eosin. × 150

FIGURE 3.—Fibrosarcoma in liver of rat fed DMN for 46 weeks. Spindle-shaped cells with elongated nuclei. Hematoxylin and eosin. × 150

FIGURE 4.—Same rat as figure 3. Lung metastasis resembling primary tumor. Hematoxylin and eosin. × 150

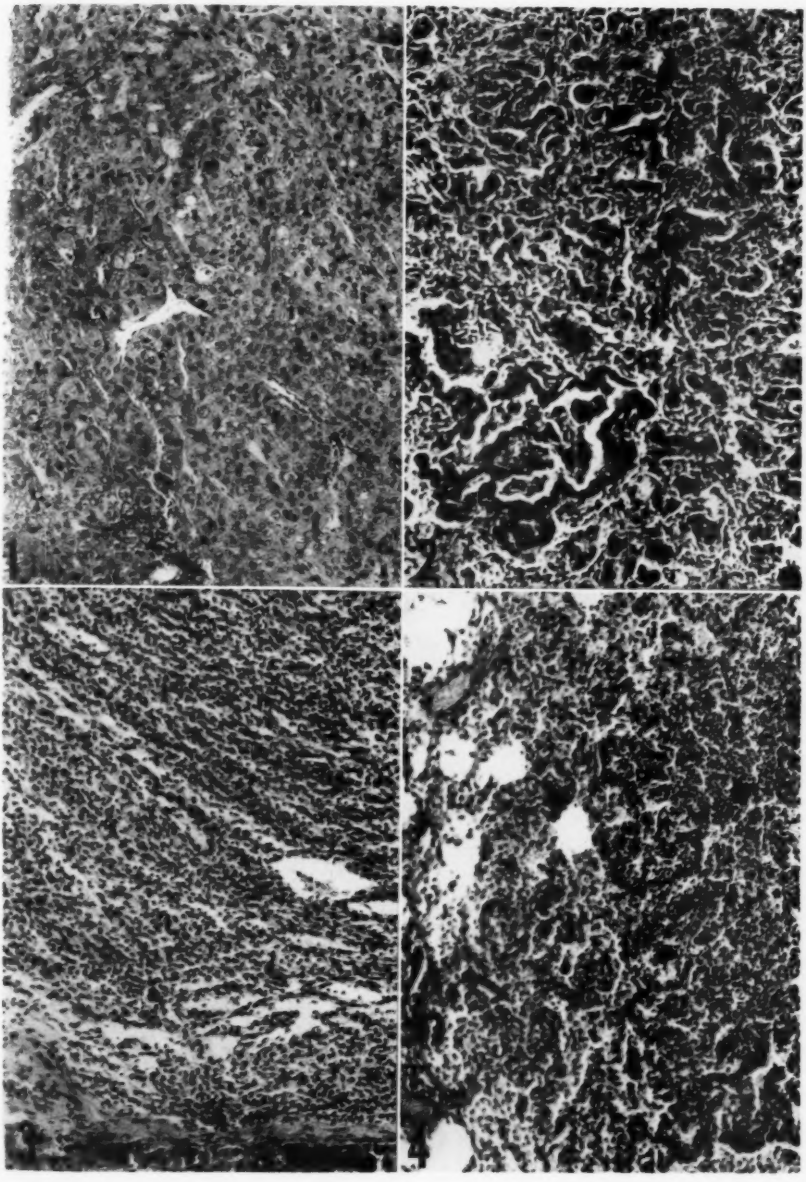


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PLATE 86

FIGURE 5.—Papillary tumor in kidney of rat fed DMN for 48 weeks. Hematoxylin and eosin. × 150

FIGURE 6.—Same rat as figure 5. Papillary tumor in lung, possibly metastatic. Mucin secretion could not be demonstrated by special stains. Hematoxylin and eosin. × 150

FIGURE 7.—Solid tumor in kidney of rat fed DMN for 31 weeks. Hematoxylin and eosin. × 150

FIGURE 8.—Same rat as figure 7. Lesion in lung shows both adenocarcinoma and squamous-cell carcinoma. Hematoxylin and eosin. × 150

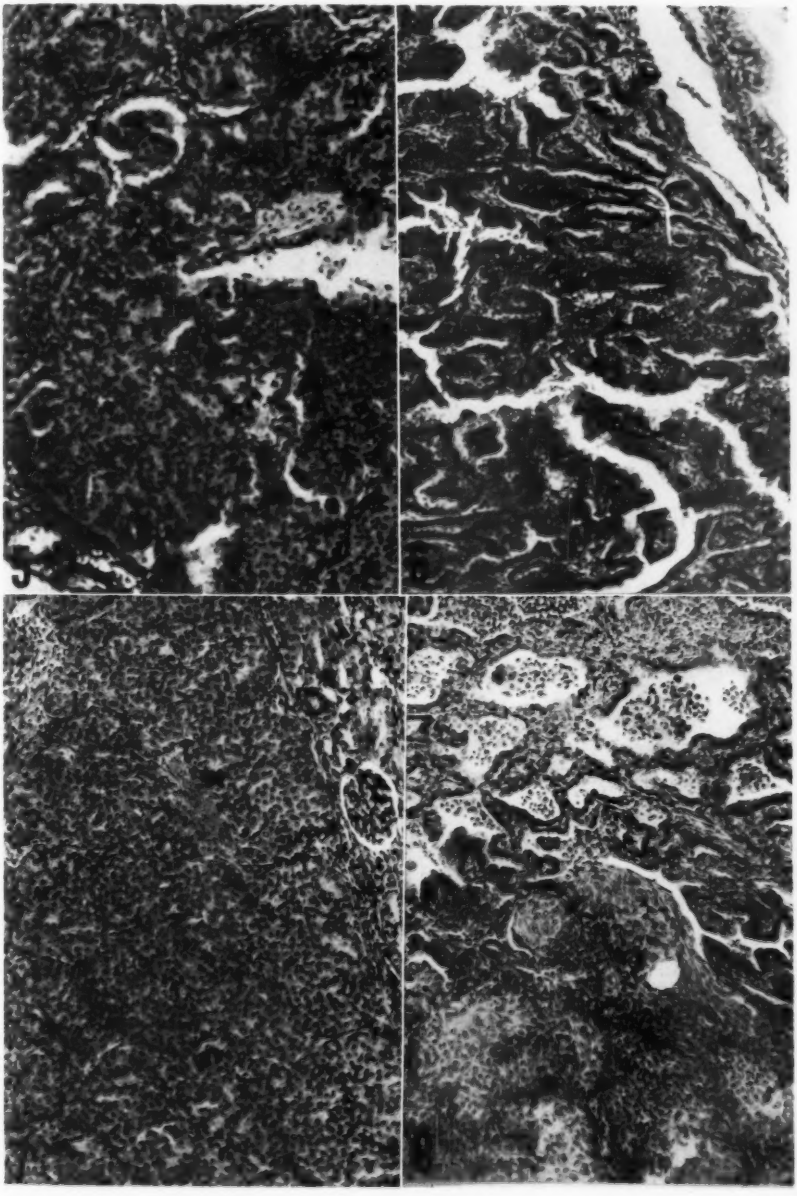

PLATE 87

FIGURE 9.—Tumor in kidney of rat fed DMN for 42 weeks. Elongated sarcomatous cells with indication of newly formed glomeruli. Hematoxylin and eosin. × 300

FIGURE 10.—Squamous-cell carcinoma in lung of rat fed DMN for 37 weeks. No other tumor found in this rat. Hematoxylin and eosin. × 150

FIGURE 11.—Squamous-cell carcinoma in lung of rat fed DMN for 48 weeks. This rat had tumors also in liver and kidney. Hematoxylin and eosin. × 150

FIGURE 12.—Epithelial tumor with alveolar structure in lung of rat fed DMN for 48 weeks. Hematoxylin and eosin. × 300

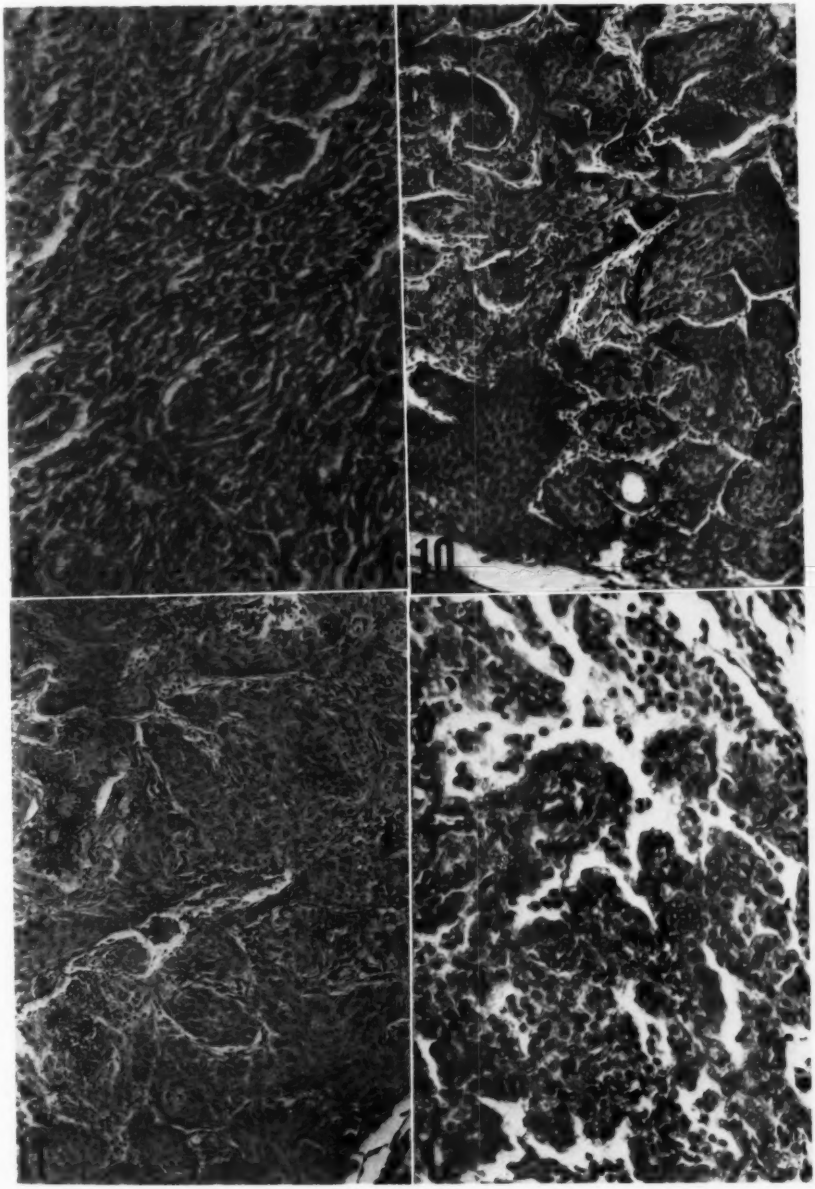

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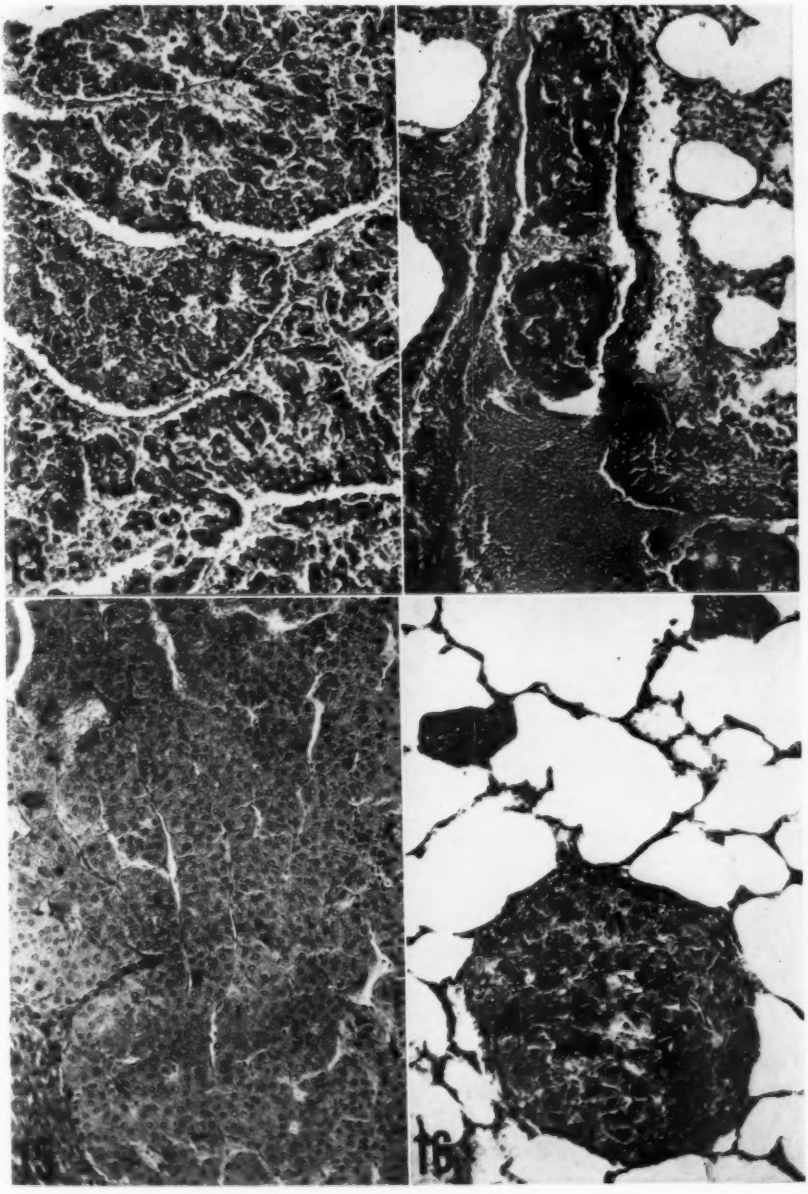
PLATE 88

FIGURE 13.—Hepatocellular carcinoma in the liver of rat fed DEN for 35 weeks. Long strands of neoplastic tissue, with invasion of veni. Hematoxylin and eosin. × 150

FIGURE 14.—Same rat as figure 13. Thrombi of liver-cell carcinoma in pulmonary artery. Hematoxylin and eosin. × 150

FIGURE 15.—Hepatocellular carcinoma in the liver of a rat fed DEN for 35 weeks. Long finger-like arrangement of neoplastic cells. Hematoxylin and eosin. × 150

FIGURE 16.—Same rat as figure 15. Tumor-cell thrombi in the pulmonary vessels with small metastatic nodule. Hematoxylin and eosin. × 150



Announcement

Manuscripts Approved for JNCI, July 15 to August 15, 1961

"Evaluation of inhibition of human tumor tissue by cancer chemotherapeutic drugs with an *in vitro* test," by Joseph A. DiPaolo and John E. Dowd.

"Chromosome studies in human leukemia. I. Acute leukemia in children," by David A. Hungerford.

"Development of spontaneous pulmonary tumors at high altitude in strain A mice," by Pablo Mori-Chavez.

"Glucose metabolism of Rous sarcoma virus-induced tumor. III. Carbohydrate metabolic enzymes in Rous sarcoma," by George Weber, Gouri Banerjee, Alvin S. Levine, and James Ashmore.

"Observations on the surface cells of the mouse omentum as studied with the phase-contrast and electron microscopes," by Marie D. Felix.

"Alternated renewal and differentiation of mucosal cells in the nondiseased part of human stomach affected with peptic ulcer and cancer," by Harald Teir and Toimi Räsämen.

"Immunohistochemical studies on the antigenic relationship of some muscle tumors to normal skeletal muscle cells," by R. Hiramoto, J. Cairns, and D. Pressman.

"Effects of adrenocorticotropin and insulin on carcinogenesis in hypophysectomized rats," by B. G. Dodge, M. A. O'Neal, J. P. Chang, and A. C. Griffin.

"Gm factors in normal γ-globulin fractions, myeloma proteins, and macroglobulins," by John L. Fahey and Sylvia D. Lawler.

"The production of viable single cell suspensions from solid tumors," by Robert E. Madden and Dean Burk.

"Site distribution of cancer deaths in husband-wife and sibling pairs," by William Y. Chen, Lyman B. Crittenden, Nathan Mantel, and W. Ross Cameron.

"The chromosome constitution of cells in effusions of cancer patients," by Takaaki Ishihara, George E. Moore, and Avery A. Sandberg.

"An electron microscopic study of a series of murine lymphoid neoplasms," by Albert J. Dalton, Lloyd W. Law, John B. Moloney, and Robert Manaker.

"Cell proliferation in precancerous liver: Relation to presence and dose of carcinogen," by Anna Kane Laird and A. D. Barton.

"Spontaneous tumors in the Sprague-Dawley rat," by S. W. Thompson, R. A. Huseby, Mary A. Fox, C. L. Davis, and R. D. Hunt.

"Observations on the occurrence of hepatomas in rainbow trout," by W. C. Hueper and W. W. Payne.

"Elimination of the effect of the A^y gene on pulmonary tumors in mice by altering its effect on normal growth," by W. E. Heston and George Vlahakis.

"Studies on the machanism of isoantigenic variant formation in heterozygous mouse tumors. I. Behavior of *H-2* antigens D and K: Quantitative absorption tests on mouse sarcomas," by Eva Klein.

"Studies on the mechanism of isoantigenic variant formation in heterozygous mouse tumors. II. Behavior of *H-2* antigens D and K. Cytotoxic tests on mouse lymphomas," by K. E. Hellström.

"Long-term cultivation in tissue culture of leukemic cells from mouse leukemia induced by Moloney virus or by X rays," by Haim Ginsburg and Leo Sachs.

"Chromosome studies in human leukemia. II. Chronic granulocytic leukemia," by Peter C. Nowell and David A. Hungerford.

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